DEVELOPMENT OF A TRANSGENIC MOUSE MODEL OF HEREDITARY PANCREATITIS

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Abstract

Development of a transgenic mouse model of hereditary pancreatitis.

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Hereditary pancreatitis, a rare autosomal dominant condition, presents with recurrent episodes of acute pancreatitis and usually progresses to chronic pancreatitis with patients at an increased risk of developing pancreatic adenocarcinoma. The majority of patients are carriers of either the R122H or N29I mutation in the cationic trypsinogen gene (*PRSS1)*, one of the most abundant digestive enzymes produced by the pancreas. The mutations are thought to increase autoactivation of the proenzyme cationic trypsinogen to trypsin within the acinar cell, initiating a digestive enzyme cascade leading to pancreatitis. A genetically modified mouse model expressing wild type (wt) or mutant human *PRSS1* may be exploited to study the molecular events in hereditary pancreatitis.

To investigate this, two transgenic mouse models were developed to either inducibly or constitutively express wt or mutant human *PRSS1* tagged with a haemaglutinin motif to aid identification. In the inducible model, transgenic animals treated with tetracycline would enable binding of OptrtTA (optimised reversed Tet transcription activator) inducing the expression of PRSS1-based transgenes specifically in the acinar cells of the pancreas. In the constitutive model, expression of PRSS1-based transgenes is driven by the acinar cell specific rat elastase I promoter. Prior to generation of transgenic strains, expression constructs were created and tested by transient transfection of human cell lines that showed expression of the recombinant proteins. In addition, enzymatic activity of tagged human PRSS1 was demonstrated using an *in vitro* trypsin assay.

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Declaration of originality

I hereby declare that this thesis has been composed by myself and has not been accepted in any previous application for a degree. The work, of which it is a record, has been done by myself except where stated. All sources of information have been acknowledged by means of references.

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Abbreviations

A	Absorbance
ACh	Acetylcholine
AP	Acute pancreatitis
APS	Ammonium Persulphate
ATP	Adenosine Triphosphate
BSA	Bovine serum albumin
Bp	Base pairs
BZiPAR	Rhodamine 110, bis-(N-CBZ-L-isoleucyl-L-prolyl-L-arginine amide), dihydrochloride)
CCK	Cholecystokinin
cDNA	Complementary Deoxyribonucleic Acid
Ci	Curies
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CP	Chronic pancreatitis
CTRC	Chymotrypsin C
DMEM	Dulbecco's modified Eagle's medium

DMSO	Dimethyl Sulphoxide
dNTP	Deoxynucleoside triphosphate
Dox	Doxycycline
ECL	Enhanced chemiluminescence
EDTA	Ethylene diaminetetracetic Acid
ER	Endoplasmic Reticulum
FBS	Foetal bovine serum
HA	Haemaglutinin
HCCl	Hexamine cobalt chloride
HEPES	N-2-hydroxyethylpiperazine-N-ethane sulphonic acid
HP	Hereditary Pancreatitis
HRP	Hoseradish Peroxidase
IL	Interleukin
IP_3	Inositol 1,4,5 triphosphate
IPTG	Isopropyl-β-D-thiogalactoside
kDa	Kilo-Daltons
kb	Kilo-bases
l	Litre(s)

LB	Luria Bertani
M	Molar / Mega-
m	milli-
μ	micro-
MODS	Multiple organ dysfunction syndrome
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
O.D	Optical density
PAGE	Polyacrylamide gel electrophoresis
PanIN	Pancreatic intraepithelial neoplasia
PBS	Phosphate buffered saline
PCI	Phenol Chloroform Isoamyl alcohol
PCR	Polymerase chain reaction
PDAC	Pancreatic ductal adenocarcinoma
PRSS1	Protease, serine 1
PSTI	Pancreatic secretory trypsin inhibitor
RO	Reverse osmosis
RPMI	Roswell Park Memorial Institute media

rtTA	Reverse tetracycline controlled transactivator
SBTI	Soybean trypsin inhibitor
SIR	Standardised incidence ratio
SIRS	Systemic inflammatory response syndrome
SDS	Sodium dodecyl sulphate
SLIP	Stuart Linn immunoprecipitation buffer
SOC	Salt optimised and carbon
SPINK1	Serine protease inhibitor, Kazal type 1
SSC	Standard saline citrate
SQ	Super-Q
TAE	Tris acetate EDTA
TAP	Trypsinogen activation peptide
TEMED	N,N,N',N'-tetramethylethylenediamine
TetR	Tet repressor
TNF-α	Tumour necrosis factor alpha
TRE	Tetracycline response element
tTA	Tetracycline controlled transactivator
Wt	Wild type

X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

IUPAC Coding

Nucleic acid codes

A	Adenine
C	Cytosine
G	Guanine
N	Any nucleotide
T	Thymine

Amino acid designation

Alanine	A	Leucine	L
Arginine	R	Lysine	K
Asparagine	N	Methionine	M
Aspartic Acid	D	Phenylalanine	F
Cysteine	C	Proline	P
Glycine	G	Serine	S
Glutamic Acid	E	Threonine	T
Glutamine	Q	Tryptophan	W
Histidine	H	Tyrosine	Y
Isoleucine	I	Valine	V

Chapter 1

1 INTRODUCTION

1.1 The Pancreas

1.1.1 Anatomy

The pancreas originates from the primitive digestive tube, which is formed by a single layer of endoderm. Two epithelial buds, referred to as the dorsal pancreas and ventral pancreas, coalesce to form a single organ (Edlund 2002). It is a retroperitoneal organ, located against the posterior abdominal wall and its anterior surface is covered by peritoneum. The pancreas weighs 60 to 150g in adults and is divided into regions that are described as the head and uncinate process, neck, body, and tail (Sinnatamby 2000). The portion of the gland that secretes pancreatic juice drains initially into small ductules that coalesce into a single duct (pancreatic duct of Wirsung), which usually joins the common bile duct to form the ampulla of Vater. The ampulla opens through the duodenal papilla, and its orifice is encircled by the sphincter of Oddi (Sinnatamby 2000).

1.1.2 Physiology

Functionally, the pancreas is a mixed endocrine-exocrine gland secreting its products straight into the blood stream or in the duodenum respectively. The endocrine pancreas is made up of hormone producing cells organised

into clusters known as islets of Langerhans dispersed throughout the pancreas (Madsen, Jensen et al. 1996). The islets are made up of four cell types (α cells, β cells, δ cells and PP cells) each producing a particular hormone (glucogen, insulin, somatostatin and pancreatic polypeptide respectively). The hormones secreted by the endocrine cells regulate nutrient metabolism, in particular, β cells produce insulin to maintain glucose homeostasis (Madsen, Jensen et al. 1996). The exocrine pancreas, which represents 85% of the gland, consists of the ductal tree and the secretory acinar cells (Ganong 2001).

1.1.3 Acinar Cell

The acinar cell, the most numerous cell type in the pancreas, is a classic protein producing and secreting cell (Palade 1975). Digestive enzymes (i.e. proteases, amylase, lipase etc) which are required for digestion of protein, carbohydrates and lipids, are synthesized as inactive precursors or proenzymes by ribosomes at the rough endoplasmic reticulum, found at the basal and lateral aspects of the acinar cell (Caro and Palade 1964). The enzymes are separated from constitutive and lysosomal proteins within the Golgi apparatus and packaged into condensing vacuoles (Fig 1.1). The enzymes are sorted further within the immature condensing vacuoles by pH dependant aggregation with exclusion of constitutive proteins, thus resulting in the formation of mature concentrated zymogen granules, which store the enzymes at the apical pole, until secretion is initiated (Douglas

and Rubin 1961). The internal milieu of the acinar cell is tightly regulated by homeostatic mechanisms in order to maintain, amongst others, a constant pH and cytosolic calcium concentration (Pandol 2005). Under physiological conditions the cytosolic calcium concentration of the acinar cell is approximately 0.1μM compared to 1mM in the extracellular fluid and 100μM in the intracellular organelles, for instance the endoplasmic reticulum (Argent, Case et al. 1982). The relative concentrations are maintained by active pumping of calcium out of the cytosol by ATP dependant transporters (Mogami, Nakano et al. 1997). Regulation of pH is less well understood, but homeostasis is largely achieved by active transportation of hydrogen ions by a H^+-ATPase pump (Muallem and Loessberg 1990). Rodent acinar cells have a cytosolic pH between 6.77 and 7.28 compared to an extracellular pH which may vary between 7.2 and 7.5 (Williams and Dimagno 2009). The pH of the secretory pathway is mildly acidic (~pH 6.8) in the Golgi complex, whilst the condensing vacuoles are more acidic (~pH 6.5) and the mature zymogen granules are neutral (Orci, Ravazzola et al. 1987). Lysosomes are acidic with a pH of 5.0 (Williams and Dimagno 2009).

The acinar cells secrete the contents of the zymogen granules in response to several neurohormonal regulators released in response to the ingestion of food (Williams 2006). The principle secretagogues, acetylcholine (ACh) and cholecystokinin (CCK), activate the inositol 1,4,5 triphosphate (IP_3) /

diacyl glycerol signalling pathway which leads to a oscillatory rise in cytosolic Ca^{2+} (Thorn, Gerasimenko et al. 1994) and triggers zymogen granule exocytosis (Tsunoda, Stuenkel et al. 1990). Other than IP_3, the secondary messengers nicotinic acid adenine dinucleotide phosphate and cyclic adenosine dinucleotide phosphate ribose also regulate Ca^{2+}-dependant exocytosis (Cancela, Van Coppenolle et al. 2002). The proenzymes travel through the ductules which merge into small ducts and finally into the main pancreatic duct, to enter the duodenum (Rinderknecht 1986). Here the enteropeptidase, enterokinase (PRSS7; OMIM 606635), secreted by the brush border of the duodenum activates trypsinogen to trypsin by proteolytic cleavage of an eight amino acid peptide, trypsin-activating peptide (TAP), from the C terminus (Rinderknecht, Engeling et al. 1974). Trypsin in turn activates the other pancreatic proteases in a similar manner (Rinderknecht 1986).

1.1.4 Ductal Bicarbonate Secretion

Once the acinar cells have secreted the proenzymes, they are carried into the duodenum in a bicarbonate rich pancreatic juice. The bicarbonate is secreted by ductal cells upon stimulation by secretin (Fernandez-Salazar, Pascua et al. 2004). Approximately two litres of isotonic, bicarbonate rich, pancreatic juice enters the duodenum daily, and has a pH of 8 – 8.5 (Stuenkel, Machen et al. 1988). This raises the pH of the acid chyme entering the duodenum, from the stomach, towards neutral and thus

creating an optimal environment for pancreatic enzymes to function (Rinderknecht 1986).

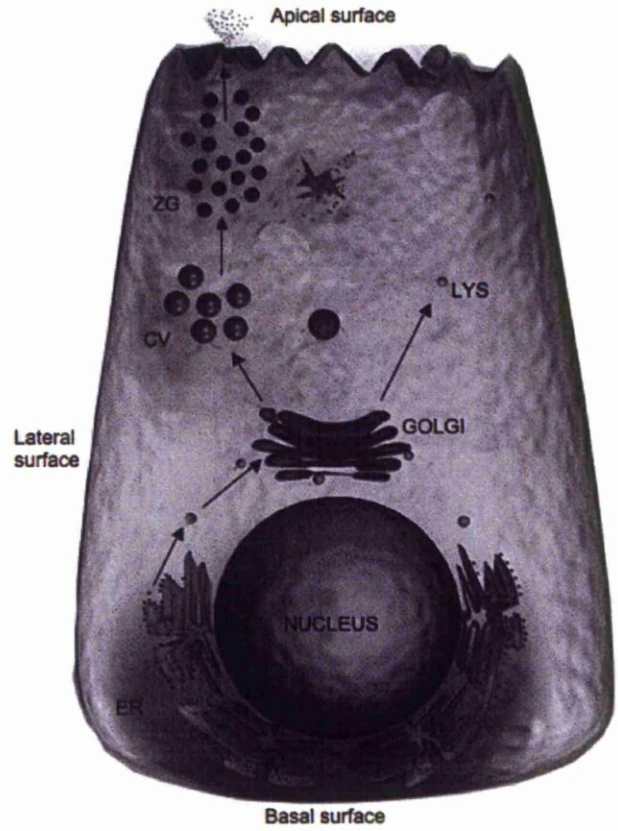

Fig 1.1 Acinar Cell

A schematic diagram of an acinar cell showing the synthesis and processing pathway of proteins from the endoplasmic reticulum (ER), through the cell. The secretory pathway comprises of immature condensing vacuoles (CV) and mature zymogen granules (ZG) for storage of proenzymes, which are released by exocytosis. A separate pathway transports enzymes to lysosomes (LYS). With permission from (van Acker, Perides et al. 2006)

1.2 Proteases

1.2.1 Pancreatic proteases

Pancreatic proteases account for approximately 80% of the enzymes, by weight, secreted by the pancreas (Whitcomb and Lowe 2007). They, along with proteases secreted at other sites in the gastrointestinal tract, are responsible for the digestion of ingested proteins to provide the body with the source for essential amino acids (Whitcomb and Lowe 2007). Pancreatic proteases can be divided into serine proteases, which are endopeptidases, and metalloproteinases (i.e. caboxypeptidases), which are exopeptidases (Whitcomb and Lowe 2007).

1.2.2 Serine Proteases

The serine protease family consists of trypsins along with elastase and chymotrypsins (Teich, Hoffmeister et al. 2000). The term arises due to the enzymes having a preserved serine residue at position 195, based on the chymotrypsin numbering system (Teich, Hoffmeister et al. 2000). To clarify the previous statement, the amino acid sequence of these enzymes can be described using the chymotrypsin numbering system or the codon numbering system. The chymotrypsin numbering system aligns all serine proteases to centre on the serine 195 residue of the chymotrypsin molecule whilst the codon nomenclature numbers the first codon (methionine) as codon 1 (Keim, Teich et al. 2000). To avoid confusion, it was agreed in 2000 that the codon system is the preferred one when describing the amino

acid positions of trypsinogen (Teich, Hoffmeister et al. 2000). The codon system will be used in this thesis unless specifically stated. Serine proteases are composed of a single peptide which folds to give a highly homologous 3D structure consisting of 2 globular domains connected by a single chain known as the autolysis loop, as shown using x-ray crystallography (Gaboriaud, Serre et al. 1996), though the specificity pocket varies from one protease to the other, in order to allow hydrolysis of particular peptide bonds (Gaboriaud, Serre et al. 1996). Trypsins hydrolyse at the site of basic amino acids (lysine and arginine), whilst the chymotrypsins hydrolyse at the site of aromatic amino acids (phenylalanine, tyrosine and tryptophan) and the elastases cleave the bond after neutral small amino acids (alanine, serine and glycine). The catalytic site is formed by histidine at position 57, aspartic acid at position 102 and serine at position 195 (chymotrypsin numbering system) and the specificity pocket is determined by the amino acids at positions 189, 216 and 226 (Craik, Largman et al. 1985) (Fig 1.2).

The serine proteases and the carboxypeptidases digest the polypeptides entering the small bowel from the stomach. As described above, the enzymes reach the small bowel in their inactive form and here trypsinogen is activated to trypsin by the action of the brush border hydrolyase, enterokinase (Rinderknecht 1986) (Fig 1.3). Typsin is considered a key

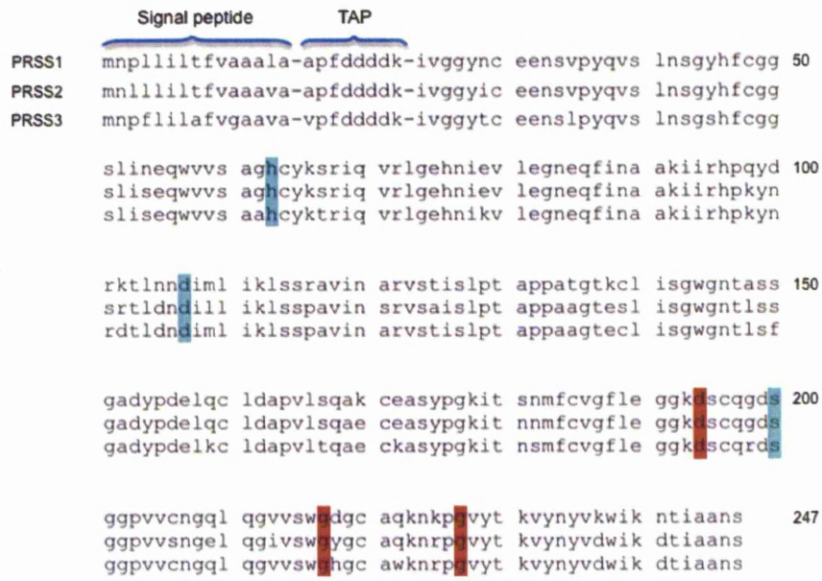

Fig 1.2 Human Trypsinogen sequences

The amino acid sequence of the three human pancreatic trypsinogens; PRSS1, PRSS2 and PRSS3. Each sequence begins with the pre-pro-peptide or signal peptide that is cleaved after transport to the ER. The proenzyme is packaged into zymogen granules ready for secretion. In the duodenum, enterokinase cleaves the trypsinogen activating peptide (TAP), converting trypsinogen to the active trypsin. The (-) shows the position of cleavage and the conserved residues within the catalytic site are highlighted in blue whilst the amino acids determining substrate specificity are highlighted red.

enzyme as it activates the proenzymes including chymotrypsinogen, proelastase and procarboxypeptidase, whilst also being able to activate trypsinogen and thus start an autocatalytic chain reaction (Rinderknecht 1986) (Fig 1.3). This property gives trypsin a central role in protein digestion and led to it being implicated in the pathophysiology of acute pancreatitis (Whitcomb, Gorry et al. 1996).

1.2.3 Trypsinogens

Trypsin and its inactive form, trypsinogen, have been well characterised and extensively studied. Trypsinogen was crystallized from bovine pancreas in 1936 (Northrop and Kunitz 1932) and later partially isolated from human pancreas by Buck *et al* in 1962 (Buck, Bier et al. 1962). It is the most abundant enzyme produced by the exocrine pancreas, making up 19% of the protein in pancreatic juice (Whitcomb and Lowe 2007). It is secreted from the pancreas as a 28 kDa polypeptide (Scheele, Bartelt et al. 1981).

There are three isoforms of trypsinogen produced within the human pancreatic acinar cell encoded by similar genetic sequences (Rowen, Koop et al. 1996) resulting in a 247 amino acid polypeptide, including a 15 amino acid signalling peptide (pre-proenzyme), and an 8 amino acid peptide (proenzyme), trypsinogen activation peptide (TAP) (Sahin-Toth 2006) (Fig 1.2).

On the basis of their isoelectric points and electrophoretic mobility, the proenzyme isoforms are known as cationic trypsinogen, anionic trypsinogen and mesotrypsinogen respectively (Scheele, Bartelt et al. 1981). Cationic trypsinogen accounts for approximately two thirds of all trypsin activity (Rinderknecht, Renner et al. 1979) and has historically been termed *trypsinogen 1* (Figarella, Clemente et al. 1969) and *trypsinogen 3* (Scheele, Bartelt et al. 1981). The cDNA was named *TYRI* (Emi, Nakamura et al. 1986) and the gene *T4* (Rowen, Koop et al. 1996) with the Unigene name and symbol of *protease, serine 1,* and *PRSS1* (OMIM 276000).

Anionic trypsinogen is the second most abundant form produced in the pancreas, making up about one third of trypsin activity (Figarella, Negri et al. 1975; Guy, Lombardo et al. 1978). It has historically been termed *trypsinogen* 2 (Figarella, Clemente et al. 1969) and *trypsinogen 1* (Scheele, Bartelt et al. 1981), whilst its cDNA was named *TYRII* (Emi, Nakamura et al. 1986) and the gene *T8* (Rowen, Koop et al. 1996). Its Unigene name and symbol are *protease, serine 2*, and *PRSS2* (OMIM 601564).

The third isoform is mesotrypsinogen (*PRSS3)*, which is secreted in relatively low amounts (Rinderknecht, Renner et al. 1984). It is, however, unique from the other two isoforms due to its resistance to trypsin inhibitors such as the Kazal type pancreatic secretory trypsin inhibitor (PSTI or by the Unigene term, serine protease inhibitor, Kazal-type, 1

(SPINK1)) and the Kunitz type soybean trypsin inhibitor (SBTI) (Rinderknecht, Renner et al. 1984; Nyaruhucha, Kito et al. 1997). This property is regarded to arise from the presence of arginine at position 198 in place of the highly conserved glycine (Szmola, Kukor et al. 2003). This change renders mesotrypsin resistant to protease inhibitors and disrupts its ability to cleave protein substrates (Szmola, Kukor et al. 2003). Interestingly, it has been observed that mesotrypsin is able to degrade trypsin inhibitors leading to speculation that its physiological role may have evolved in diets rich in these inhibitors (Sahin-Toth 2005).

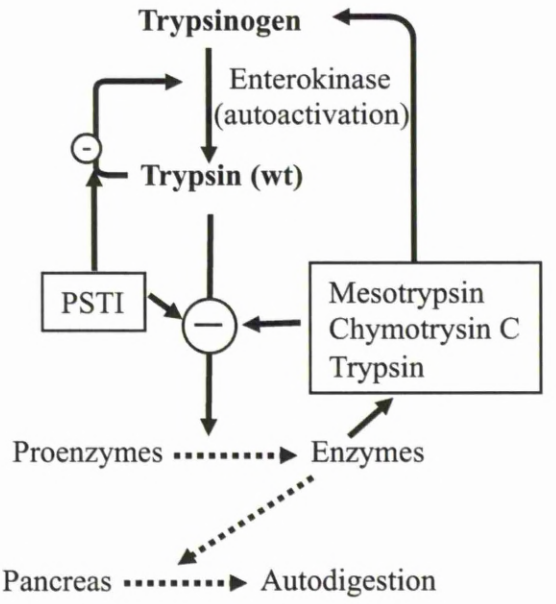

Fig 1.3 Activation of Trypsinogen

In the intestine trypsinogen is activated by enterokinase into trypsin, which can then activate other proenzymes to aid digestion of proteins in the diet. Within the cell activation of trypsinogen is inhibited by storage within zymogen granules, and tight control of intracellular calcium and pH levels. Pancreatic secretory trypsin inhibitor (PSTI or SPINK1) within the cell can inhibit 20% of activated trypsin. Inappropriate intracellular activation of trypsinogen that overwhelms these protective machanism may cause a chain reaction leading to acute pancreatitis. With permission from (Whitcomb, Gorry et al. 1996)

Most of the genes (eight) encoding for the members of the trypsinogen family are located on the long arm of chromosome 7q35 with the exception of the mesotrypsinogen gene, which is located on chromosome 9p13 (Rowen, Koop et al. 1996; Rowen, Williams et al. 2005). The genes for PRSS1 and PRSS2 lie near the 3' end of the T-cell receptor beta locus (Rowen, Koop et al. 1996), flanking two pseudogenes and also trypsinogen C, which may be functional, but no corresponding cDNA has been identified. These five trypsinogens are highly homologous with a sequence homology of at least 90% and consisting of 5 exons each spanning ~3.6kb (Rowen, Koop et al. 1996). A further two pseudogenes and a relic gene lie at the 5' end of the locus (Rowen, Koop et al. 1996).

1.2.4 Trypsinogen Activation and Degradation

The first evidence of enzyme activity in pancreatic juice came from the laboratory's of Ivan Pavlov, recipient of the Noble prize in medicine or physiology in 1904. Pavlov established a method of constructing permanent pancreatic fistulae in dogs by stitching a patch of duodenum, on which the pancreatic duct opens, onto the abdominal wall (Pavlov). In this way, whenever the dog was fed a flow of pancreatic juice was obtained, trickling from the duct over the small patch of mucous membrane and into a funnelled receiver tied to the belly of the animal.

The experiments to assess proteolytic activity of the pancreatic juice, especially with regards to trypsin, were at odds with earlier work by Pavlov

and his pupil. The reason for this became apparent when Chepovalnikow showed that the pancreatic juice obtained by Pavlov's procedure contained only small portions of trypsin, but a large amount of trypsinogen, and this latter substance needed for its conversion into trypsin the interaction of a body contained in the succus entericus (Chepovalnikov 1899), and called by Pavlov 'enterokinase'. According to Pavlov, enterokinase acts as a 'ferment of ferments', converting trypsinogen into trypsin just as trypsin converts other enzymes into their activated products (Pavlov).

Délézenne, working in the Pasteur institute, found that pancreatic juice drawn with a cannula in the duct, so to avoid contact with the patch of mucosa membrane forming the floor of the fistula, was free from trypsin (Delezenne 1901). He also proposed a different explanation of the action of enterokinase on trypsinogen from the hypothesis of Pavlov. Based on the interaction between antibody and the complement, Délézenne regarded enterokinase binding trypsinogen, thus creating the activated enzyme. Further work in the field demonstrated that, consistent with Pavlov's hypothesis, enterokinase cleaves a protein moiety resulting in the activation of trypsin (Bayliss and Starling 1905; Mellanby and Woolley 1912).

Early work to describe the biochemical characteristic of cationic and anionic trypsinogen relied on purification of these enzymes from native pancreatic juice or autolysed pancreatic extracts (Guy, Lombardo et al. 1978).

These studies indicated that the catalytic and proteolytic properties of the two human trypsins were equivalent (Colomb, Guy et al. 1978). Furthermore, both cationic and anionic trypsinogen were found to be susceptible to spontaneous autocatalytic activation (autoactivation) at pH 8 and the rate of autoactivation increased with increasing calcium concentration (Colomb, Figarella et al. 1979). Cationic trypsinogen was reported to autoactivate faster than anionic trypsinogen in all conditions except at pH 5.6 and 10mM Ca^{2+}, where it was found to be stable for a prolonged period (Colomb and Figarella 1979). In the absence of calcium at pH 5.6 cationic trypsinogen was seen to rapidly autoactivate (Colomb, Figarella et al. 1979).

Trypsin activity was analysed over an acidic pH range of 3.0-2.0 and cationic trypsin activity was found to be unaffected whilst anionic trypsin activity receded with increasing acidity. At a constant pH 8 and the absence of calcium both trypsin activities were observed to rapidly degrade (Colomb, Guy et al. 1978) suggesting trypsin-mediated degradation or autolysis (Sahin-Toth 2000). High levels of calcium stabilised cationic trypsin activity, but anionic trypsin was only 40% active at 2 hours (Colomb, Guy et al. 1978).

More recently, recombinant expression of human trypsinogen has allowed renewed study of the catalytic properties of cationic and anionic trypsin. Most of the work originated in the laboratories of Miklós Sahin-Tóth on

recombinant trypsinogen expressed in *Escherichia Coli* as denatured inclusion bodies (Sahin-Toth 2000; Sahin-Toth and Toth 2000). The proteins were renatured by an *in vitro* refolding procedure and then purified to homogeneity by affinity chromatography using immobilised ecotin, a broad specificity protease inhibitor (Lengyel, Pal et al. 1998). Zymogens were activated by bovine enterokinase (Sahin-Toth 2000; Sahin-Toth and Toth 2000) and cationic trypsin produced by recombinant techniques demonstrated similar catalytic activity to that isolated from pancreatic juice (Szilagyi, Kenesi et al. 2001). The recombinant proteins did have structural differences, compared to native proteins, primarily arising from post-translational modifications (Kiraly, Guan et al. 2006). Aminopeptidases present in the cytoplasm of *E.Coli* were observed to trim the N terminus of the recombinant trypsinogen (Sahin-Toth 2000; Kiraly, Guan et al. 2006). In human acinar cells trypsinogen was originally described as undergoing post-translational phosphorylation on Tyr 154 (Gaboriaud, Serre et al. 1996), however, recently it was shown to be sulphation (Sahin-Toth, Kukor et al. 2006), but neither modification is present in recombinant trypsinogen from *E.Coli.*

Using these techniques, cationic and anionic trypsinogen reaction kinetics have been assessed. Catalytic properties of cationic and anionic trypsin were reported to be similar (Kukor, Toth et al. 2003). Autoactivation of anionic trypsinogen at pH 8 was only observed in significant amounts

when the calcium concentration was 0.5mM or higher, whereas cationic trypsinogen was seen to autoactivate even in the absence of calcium and the effect potentiated with the addition of as little as 10μM of calcium (Kukor, Toth et al. 2003). The lack of autoactivation of anionic trypsinogen at low calcium concentrations appeared to be a consequence of rapid zymogen degradation into small peptides, whilst the addition of calcium allowed some stabilisation of the zymogen and thus an opportunity to autoactivate to trypsin (Kukor, Toth et al. 2003).

At pH 5 autoactivation of anionic trypsinogen was slower over the whole range of calcium concentrations with only 30% of total potential trypsin activity seen. This appeared to be due to stabilisation of the zymogen by acidic conditions (Kukor, Toth et al. 2003). In contrast, cationic trypsinogen autoactivated more rapidly at pH 5 in the absence of calcium than at pH 8 (Sahin-Toth 2001) with calcium-dependant stimulation seen up to 1mM concentration.

In contrast to earlier studies, high calcium concentrations (≥2mM) appeared to have an inhibitory effect on autoactivation of cationic trypsinogen (Kukor, Toth et al. 2003).

In summary, cationic and anionic trypsinogen readily autoactivated but cationic trypsinogen was able to autoactivate in the absence of calcium and

more prominently than anionic trypsinogen in acidic conditions (Kukor, Toth et al. 2003).

Comparison of the rate of autolysis demonstrated a 11-fold difference between the rapid degradation of anionic trypsin, with respect to cationic trypsin, at pH 8 and in the absence of calcium (Kukor, Toth et al. 2003). Addition of calcium reduced the rate of autolysis for both proteins, whilst interestingly, addition of sodium chloride had the effect of stabilising cationic trypsin (Kukor, Toth et al. 2003). Autolysis was thought to occur by cleavage of the Arg122-Val123 bond followed by rapid proteolysis at multiple cleavage sites (Kukor, Toth et al. 2002). This was shown to be surprisingly incorrect when it was demonstrated that trypsin-mediated cleavage of the Arg122-Val123 bond did not lead to complete degradation but instead resulted in an equilibrium between cleaved and uncleaved cationic trypsinogen due to a trypsin catalysed resynthesis of this bond (Kukor, Toth et al. 2002). This reaction was calcium sensitive, with the equilibrium favouring cleavage in the absence of calcium, at pH 8, and the rate of cleavage reduced 20-fold by addition of 5mM calcium.

An unidentified serine protease, named enzyme Y, had been attributed with trypsinogen degrading properties when a contaminant was found to degrade all trypsinogen isoforms (Rinderknecht, Adham et al. 1988). This property of enzyme Y was inhibited by millimolar concentrations of calcium suggesting it may have a protective role in the event of

intrapancreatic trypsin activation (Rinderknecht, Adham et al. 1988). A candidate for this elusive enzyme Y was not identified for almost 20 years, when it was serendipitously identified as being the same as serum calcium decreasing factor (caldecrin), now commonly known as chymotrypsin C (CTRC) (Nemoda and Sahin-Toth 2006). Chymotrypsin C was found to degrade all three human trypsinogens in a calcium-dependant manner (Szmola and Sahin-Toth 2007) whilst at higher calcium concentrations (>1mM) chymotrypsin C removed the N-terminal tripeptide from cationic trypsinogen resulting in a 3-fold increase in autoactivation (Nemoda and Sahin-Toth 2006).

1.2.5 Protective Mechanisms Within the Acinar Cell

A number of protective mechanisms exist to protect the acinar cell from premature enzyme activation. The enzymes are produced as inactive zymogens that can only be activated in the duodenum by the action of enterokinase cleaving trypsinogen to trypsin, which then activates the other proenzymes (Neurath and Walsh 1976; Rinderknecht 1986). The zymogens are packaged into membrane bound vesicles and are not allowed to be free within the cytoplasm (Sutton, Criddle et al. 2003), and the cell also contains a serine protease inhibitor, Kazal type 1 (SPINK1) which can inhibit 20% of potential trypsin activity (Witt, Luck et al. 2000; Nathan, Romac et al. 2005). Chymotrypsin C is thought to be a second line of defence within the acinar cell given its ability to degrade all three trypsin

isoforms at low calcium concentrations (Szmola and Sahin-Toth 2007). Trypsin can deactivate itself through a process of autolysis, given the correct conditions (Sahin-Toth 2001). Calcium concentrations and pH are tightly regulated within the organelles of the acinar cell (Sherwood, Prior et al. 2007) If these mechanisms are defective or overwhelmed then inappropriate intra-acinar activation of trypsinogen can lead to cellular injury and acute pancreatitis.

1.3 Acute Pancreatitis

1.3.1 Epidemiology

Acute pancreatitis is a common clinical condition presenting with abdominal pain of varying severity, usually localised to the epigastrium. Nausea and vomiting are associated symptoms and diagnosis is confirmed by raised plasma concentrations of pancreatic enzymes. The incidence is 150 to 450 cases per million population, and appears to be rising in the UK (Corfield, Cooper et al. 1985; McKay, Evans et al. 1999; Toh, Phillips et al. 2000). The incidence may be higher in other European countries (Yadav and Lowenfels 2006). The mean or median age of onset in most studies was the sixth decade of life, with incidence increasing with age (Yadav and Lowenfels 2006). Though the overall sex distribution varied between studies, gallstone induced acute pancreatitis was more common in female patients whilst alcoholic acute pancreatitis was more common in males (Yadav and Lowenfels 2006). In most cases the disease follows a mild

course with a mortality of less than 1%. However, 20% of patients will follow a severe course, and 10 – 30% of those with severe acute pancreatitis die (Uhl, Warshaw et al. 2002). An improved understanding of the natural history of the disease, with early identification of complication, advances in intensive care and avoidance of major surgery have resulted in an overall fall in mortality rate from 25-30% to 6-10% (Neoptolemos, Raraty et al. 1998).

1.3.2 Aetiology

Acute pancreatitis occurs secondary to biliary disease in half the cases and 20 – 25% as a result of excessive alcohol consumption (Johnson, Charnley et al. 2005). Other causes include metabolic disturbance (i.e. hypercalcaemia, hypertriglyceridaemia), drugs (i.e. glucocorticoids, azothioprine, thiazides, and oestrogens), duct obstruction (i.e. secondary to tumour or pancreatic divisum), iatrogenic (i.e. endoscopic retrograde cholangiopancreatography), genetic (i.e hereditary pancreatitis) and trauma (Kingsnorth and O'Reilly 2006). However, 20% of cases in adults remain idiopathic (Yadav and Lowenfels 2006), though many may be due to microlithiases (Lee, Nicholls et al. 1992) or genetic predisposition (Whitcomb 2004).

1.3.3 Pathogenesis

Despite the variety of aetiological factors that may potentially initiate acute pancreatitis, the clinical course is indistinguishable and appears

independent of the original stimulus. It has been postulated that acute pancreatitis is an autodigestive process occurring as a result of premature activation of the zymogens within the pancreas resulting in cellular injury (Steer and Meldolesi 1988; Leach, Modlin et al. 1991; Mithofer, Fernandez-del Castillo et al. 1998). The acinar cell injury leads to a local inflammatory reaction, with the release of pro- and anti-inflammatory mediators that may determine progression to a systemic inflammatory response syndrome (SIRS) (Davies and Hagen 1997; Brady, Christmas et al. 1999; Raraty, Connor et al. 2004). It is this systemic response that, if sufficiently severe, can lead to multiple organ dysfunction syndrome (MODS) that is ultimately responsible for the early mortality seen within the first week of illness (Neoptolemos, Raraty et al. 1998; Wilson, Manji et al. 1998; McKay, Evans et al. 1999). There is still considerable debate as to the exact mechanism of premature zymogen activation within the acinar cells and the sequence leading to MODS (Halangk and Lerch 2005). However, inappropriate activation of intra-acinar trypsinogen is considered to be a central early event in the pathogenesis of acute pancreatitis, and therefore the regulation of inactive trypsinogen within the acinar cell is of utmost importance.

1.3.4 Trypsinogen Autoactivation

Trypsinogen is considered a critical player in the early events of acute pancreatitis, as its premature conversion to trypsin can initiate an enzyme

cascade leading to cellular injury (Neoptolemos, Kemppainen et al. 2000; Whitcomb 2001). Human cationic trypsinogen is unique in its ability to autoactivate (Figarella, Miszczuk-Jamska et al. 1988) and this property makes it a prime candidate for causing pancreatitis (as discussed above). Further evidence from studies of hereditary pancreatitis (HP) tends to support this theory of autoactivation. Patients with point mutations in the cationic trypsinogen gene have a clinical history of early onset recurrent acute pancreatitis (Whitcomb, Gorry et al. 1996; Gorry, Gabbaizedeh et al. 1997). *In vitro* studies have shown that the two common mutations increase the ability of trypsinogen to autoactivate (Sahin-Toth 2001), thus suggesting this as a key initiating event. However, doubt remains with regards to premature intrapancreatic trypsinogen autoactivation as the initial event in acute pancreatitis (Halangk, Kruger et al. 2002), with the role of cathepsin B (Halangk, Lerch et al. 2000) or other lysosomal enzymes being proposed as the culprits.

1.3.5 Co-localisation

It has also been proposed that Cathepsin B, a lysosomal hydrolase, is responsible for premature trypsinogen activation. This theory known as 'co-localisation' proposes that mis-sorting of cathepsin B into the secretory pathway is the critical event (Saluja, Saito et al. 1985; Saluja, Hashimoto et al. 1987), and is based on observations that trypsin and cathepsin B are both present within cytoplasmic vacuoles during the early stages of acute

experimental pancreatitis (Watanabe, Baccino et al. 1984) and that cathepsin B can activate trypsinogen *in vitro* (Figarella, Miszczuk-Jamska et al. 1988). Alternatively, co-localisation has been suggested as a protective feature (Gorelick and Matovcik 1995). A problem with the co-localisation hypothesis is that cathepsin B has been shown to be a normal passenger in the secretory pathway indicating that co-localisation occurs under normal circumstances (Kukor, Mayerle et al. 2002). *In vivo* studies utilising lysosomal inhibitors failed to prevent the onset of experimental acute pancreatitis (Lerch, Halangk et al. 2000) and a knockout mouse model of cathepsin B null ($ctsb^{-/-}$) animals demonstrated a significantly reduced trypsin activity after secretagogue induced pancreatitis, however, total TAP levels were only reduced by 50% and the level of pancreatitis and systemic inflammatory response were not drastically altered (Halangk, Lerch et al. 2000).

1.3.6 Calcium Signalling in Acute Pancreatitis

The calcium ion is an intracellular messenger that is responsible for normal zymogen secretion from the apex of the acinar cell (Sutton, Criddle et al. 2003). Calcium concentration within the cytoplasm of the acinar cell is low compared to the extracellular fluid and intracellular stores (i.e the endoplasmic reticulum) (Mogami, Tepikin et al. 1998; Petersen, Gerasimenko et al. 1998) and these concentration gradients are maintained by active transport of calcium driven by ATPase pumps (Bayerdorffer,

Streb et al. 1984) to maintain tight control of calcium levels in differing parts of the cell. Regional increase in calcium concentration can act as a secondary messenger to control events, including exocytosis of zymogen granules into the duct lumen (Criddle, McLaughlin et al. 2007). Under physiological conditions, CCK or Ach generate oscillatory calcium signals confined to the apical pole of the acinar cell with spread to the basolateral surface limited by calcium uptake into the intervening mitochondria (Mukherjee, Criddle et al. 2008). Supramaximal stimulation of the acinar cell with CCK or its analogue caerulein in rodents results in a different pattern of calcium signalling, a sustained global rise in cytosolic calcium levels as opposed to oscillatory rises seen with physiological doses, initiating intracellular zymogen activation, cytoskeletal disruption, loss of secretory polarity, vacuolisation and other features characteristic of acute experimental pancreatitis (Raraty, Ward et al. 2000). Many causes of acute pancreatitis have been shown to elicit these pathologically sustained global elevations of cytosolic calcium (Criddle, Gerasimenko et al. 2007).

1.4 Chronic Pancreatitis

Chronic pancreatitis is a persistent inflammatory condition characterised by permanent damage to the structure and function of the pancreas gland, leading to exocrine and endocrine insufficiency usually associated with repeated attacks of severe abdominal pain (Truninger, Ammann et al. 2001). The incidence is approximately 3-10 per 100,000 population per

year (Pezzilli 2009). It is more common in males compared to females with a ratio of 4:1(Etemad and Whitcomb 2001). In the industrialised world, alcohol is the major aetiological factor, accounting for 70-80% of all cases (Truninger, Ammann et al. 2001) with the remainder having no obvious cause and being described as idiopathic chronic pancreatitis. Smoking has recently been implicated as an independent risk factor for developing chronic pancreatitis (Yadav, Hawes et al. 2009).

Advances in genetic testing led to the identification of mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) (Cohn, Friedman et al. 1998; Sharer, Schwarz et al. 1998), *SPINK1* (Witt, Luck et al. 2000), cationic trypsinogen (Whitcomb, Gorry et al. 1996) and Chymotrypsin C (Rosendahl, Witt et al. 2008) genes in patients with idiopathic chronic pancreatitis. A mutation of the anionic trypsinogen gene, resulting in substitution of glycine to arginine at codon 191 (G191R), was found to be over-expressed in the control population (3.4%) compared to patients with chronic pancreatitis (1.3%) (Witt, Sahin-Toth et al. 2006). The G191R mutation introduces a new tryptic cleavage site, and biochemical analysis demonstrated a complete loss of trypsin activity, suggesting a protective role against chronic pancreatitis (Witt, Sahin-Toth et al. 2006). Mutations in *CFTR* and *SPINK1* have been described as 'disease modifiers', either lowering the threshold for initiating pancreatitis or exacerbating the severity of the disease (Pfutzer, Barmada et al. 2000).

No significant association exists between alcoholic chronic pancreatitis and genes encoding for the detoxifying enzymes glutathione S-transferases (GSTM1, GSTT1, GSTP1), the cytochrome P450 (CYP2E1, CYP1A1) enzymes (Burim, Canalle et al. 2004), and with polymorphisms in genes encoding for TGF-b1, interleukin-10, interferon-γ, alcohol dehydrogenase 3 or CYP2E1 (Schneider, Barmada et al. 2004).

Chronic pancreatitis has been associated with an increased risk of developing pancreatic cancer (Howes, Lerch et al. 2004; Raimondi, Lowenfels et al. 2010), though the pathway for such a process remains to be elucidated. The risk of developing pancreatic adenocarcinoma in patients with chronic pancreatitis has been estimated to be in the order of 5% at 20 years from disease onset (Lowenfels, Maisonneuve et al. 1993; Goldacre, Wotton et al. 2008). The clearest evidence for such a link has come from studies of patients with hereditary pancreatitis as a result of mutations in the PRSS1 gene (Lowenfels, Maisonneuve et al. 1997).

1.4.1 SPINK1

The most frequently found mutation, asparagine to serine substitution at codon 34 (N34S) (Witt, Luck et al. 2000), described in *SPINK1* is associated with idiopathic chronic pancreatitis in over 20% patients (Witt, Luck et al. 2000) and with alcoholic chronic pancreatitis in 6% (Witt, Luck et al. 2001). However, this mutation was found in up to 50% of patients from Southern India diagnosed with tropical pancreatitis, a condition

previously thought to be caused by malnutrition (Bhatia, Choudhuri et al. 2002; Chandak, Idris et al. 2002). The N34S mutation does not segregate with the disease to suggest an autosomal dominant or recessive pattern of inheritance, and given that it is relatively common in the general European population (1-2%), the pathogenesis appears to be more complex (Etemad and Whitcomb 2001).

A rare allele, c.27delC, where a frameshift produces a stop codon and the entire SPINK1 gene is deleted, was found in a family with hereditary chronic pancreatitis with a penetrance of 75% (Le Marechal, Chen et al. 2004). However, in another family, the penetrance was a much lower 29% (Le Marechal, Chen et al. 2004). Mutations in the signal peptide, which result in impaired secretion of SPINK 1, have also been shown to be associated with hereditary chronic pancreatitis (Kiraly, Boulling et al. 2007). These families provide some evidence for haploinsufficiency as a pathogenic mechanism for chronic pancreatitis (Le Marechal, Chen et al. 2004; Kiraly, Boulling et al. 2007).

1.4.2 CFTR

Cystic fibrosis (CF) is a common autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator which encodes a cAMP activated chloride channel in epithelial cell membranes (Riordan, Rommens et al. 1989). The relationship between *CFTR* mutations and the mechanism causing chronic pancreatitis remains

poorly understood (Chen and Ferec 2009). This is compounded by a high number of mutations that have been described in the *CFTR* gene (Cohn, Friedman et al. 1998) . Interestingly, it appears as though it is the less severe CF-causing mutations that maintain a higher percentage of pancreatic sufficiency, which are associated with an increased risk of chronic pancreatitis (Cohn, Neoptolemos et al. 2005). A German study highlighted the importance of CFTR mutation in sporadic CP by analysing 67 patients with idiopathic chronic pancreatitis with 60 matched controls and found that abnormal CFTR alleles were twice as common in patients with idiopathic chronic pancreatitis (Weiss, Simon et al. 2005).

1.4.3 Chymotrypsin C

Chymotrypsin C is considered a second line of defence against prematurely activated intrapancreatic trypsin (Szmola and Sahin-Toth 2007) and two studies investigating idiopathic chronic pancreatitis demonstrated a significantly higher incidence of loss of function mutations in patients. (Masson, Chen et al. 2008; Rosendahl, Witt et al. 2008). Biochemical analysis of some of the missense mutations indicated that they caused impaired activity and/or reduced secretion, thus indicating that CTRC is a pancreatitis susceptibility gene (Rosendahl, Witt et al. 2008).

1.5 Hereditary Pancreatitis

Hereditary pancreatitis was first described by Comfort *et al* in 1952 when working at the Mayo clinic (Comfort and Steinberg 1952). They described

a family with four definite and two suspected cases of relapsing chronic pancreatitis in childhood/adolescence (Comfort and Steinberg 1952). The observed pattern of inheritance appeared to follow an autosomal dominant mode but with an incomplete penetrance (Comfort and Steinberg 1952). This observation has since been confirmed by other groups in Europe and North America (Gross and Comfort 1957; Gross 1958; Gross, Ulrich et al. 1962; Gerber 1963; Sibert 1978; Le Bodic, Schnee et al. 1996; Sossenheimer, Aston et al. 1997; Amann, Gates et al. 2001; Keim, Bauer et al. 2001). Indeed, the incomplete penetrance has been noted by various groups with a figure of 80% penetrance being widely accepted (Howes, Lerch et al. 2004). However, as affected individuals are more likely to be tested for the mutation than unaffected individuals and families with few affected members are less likely to be recruited, estimates of penetrance may be overestimated (Howes, Lerch et al. 2004). A French study, which has described a national exhaustive series of hereditary pancreatitis, found that the penetrance of the PRSS1 mutations was 93% by including patients with only morphological pancreas alterations (Rebours, Boutron-Ruault et al. 2009).

Hereditary pancreatitis accounts for approximately 1% of all cases of pancreatitis (Whitcomb 2004). It is characterized by the onset of recurrent attacks of acute pancreatitis in childhood and frequent progression to chronic pancreatitis (Moir, Konzen et al. 1992; Konzen, Perrault et al.

1993; Perrault 1994) The classic clinical and demographic characteristics include recurrent episodes of pancreatitis during childhood, equal gender distribution, the frequent presence of pancreatic duct stones, a positive family history, and the absence of other known causes of pancreatitis (Kattwinkel, Lapey et al. 1973; Sato and Saito 1974; Konzen, Perrault et al. 1993). The EUROPAC definition of hereditary pancreatitis is two or more first-degree relatives, or three or more second-degree relatives, in two or more generations with recurrent acute pancreatitis and/or chronic pancreatitis in the absence of other precipitating or causative factors such as gallstones, tropical pancreatitis or excess ethanol consumption (Howes, Lerch et al. 2004). Cases where these criteria were not met, but there was more than one affected family member, were classified as familial chronic pancreatitis (Howes, Lerch et al. 2004). Other groups have used slightly different definitions when categorising patients as suffering from hereditary pancreatitis (Teich, Mossner et al. 1998).

The aetiology of hereditary pancreatitis had remained obscure for almost 50 years until the application of modern molecular genetic techniques. By employing linkage analysis using satellite markers, three groups established segregation between the disease phenotype and the long arm of chromosome 7 (Le Bodic, Bignon et al. 1996; Pandya, Blanton et al. 1996; Whitcomb, Preston et al. 1996). Once the hereditary pancreatitis gene was mapped to 7q35, positional cloning using a candidate gene approach was

employed, whereby genes already known to be in that region were sequenced (Whitcomb, Gorry et al. 1996). Soon afterwards Whitcomb *et al* identified a mutation in the third exon of the cationic trypsinogen gene on chromosome 7q35 which segregated with the disease in five families (Whitcomb, Gorry et al. 1996).

1.5.1 Mutations in the cationic trypsinogen gene

Since identification of PRSS1 as a disease gene, a number of different mutations have been identified. The two most frequently occurring mutations in HP are R122H (approximately 70%) and N29I (approximately 20%) (Sahin-Toth 2006) (Fig 1.4). They have been identified in families with hereditary pancreatitis from Europe (Ferec, Raguenes et al. 1999; Teich, Mossner et al. 1999; Witt, Luck et al. 1999; Howes, Lerch et al. 2004), Japan (Nishimori, Kamakura et al. 1999), the UK (Bell, Bennett et al. 1998; Creighton, Lyall et al. 1999), the USA (Whitcomb, Gorry et al. 1996; Gorry, Gabbaizedeh et al. 1997) and Australia (McGaughran, Kimble et al. 2004). However, these mutations in PRSS1 were not found in two hereditary pancreatitis families from Brazil (Bernardino, Guarita et al. 2003) and to date no hereditary pancreatitis families have been reported from Africa.

The R122H mutation was easily identified as it created a novel recognition site for the restriction endonuclease *Afl*III. However, it was demonstrated that a neutral polymorphism within this enzyme recognition site may

produce a false negative result (Howes, Greenhalf et al. 2001). An alternative mutation specific polymerase chain reaction approach was therefore developed for detection of the mutation even in the presence of the polymorphism (Howes, Greenhalf et al. 2001).

1.5.2 R122H

The R122H mutation remains the most frequent genetic alteration associated with hereditary pancreatitis (Sahin-Toth 2006). After its discovery in 1996, Whitcomb proposed that it disrupted the self-destruct mechanism that allows autolysis of prematurely activated intrapancreatic trypsin (Whitcomb, Gorry et al. 1996) (Fig 1.4). Loss of this 'fail-safe' mechanism in the R122H mutant (referred to as 'supertrypsin') would result in increased intrapancreatic trypsin activity, which could activate other proenzymes, leading to the onset of acute pancreatitis (Whitcomb 2001).

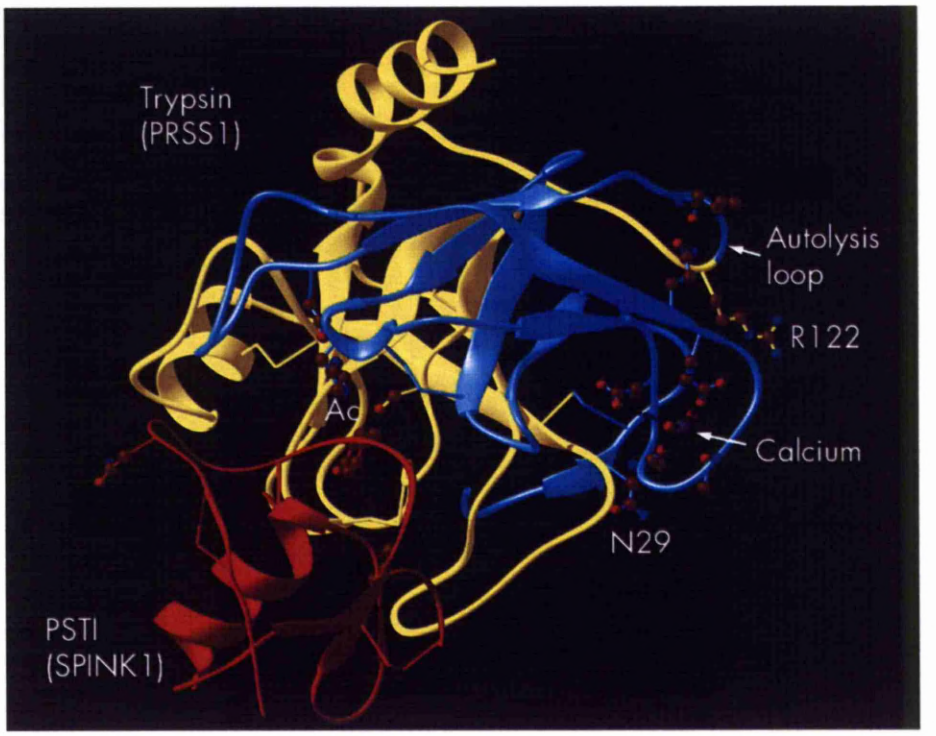

Fig 1.4 Cationic Trypsin Mutations

A schematic ribbon diagram showing cationic trypsin and the positions of the R122H and N29I mutations with respect to the autolysis loop and the calcium binding pocket. SPINK1 is shown causing inhibition by binding to the activation (AC) site of trypsin. With permission from (Whitcomb, Gorry et al. 1996)

The R122H mutation is a single guanine (G) to adenine (A) transition mutation in the third exon of PRSS1 that results in an arginine (CGC) to histidine (CAC) missense substitution at amino acid residue 122 (Whitcomb, Gorry et al. 1996). Originally the mutation was referred to as R117H based on the chymotrypsinogen numbering system but later revised in accordance with the nomenclature working group (Teich, Hoffmeister et al. 2000).

1.5.3 N29I

A second mutation in PRSS1 was discovered a year later in two affected families without the R122H mutation (Gorry, Gabbaizedeh et al. 1997). A single adenine (A) to thymine (T) transversion mutation was identified in exon 2 that results in a change from asparagine (AAC) to isoleucine (ATC) substitution at amino acid 29 (Gorry, Gabbaizedeh et al. 1997). The mutation was originally known as N21I according to the chymotrypsinogen numbering system but later revised to N29I (Teich, Hoffmeister et al. 2000).

1.5.4 A16V

The third most frequent mutation, where there is a cytosine (C) to thymine (T) missense mutation, has been identified in exon 2 that leads to an alanine (GCC) to valine (GTC) substitution at codon 16 (A16V)(Witt, Luck et al. 1999). This mutation affects the first amino acid of the trypsinogen molecule and thus directly the cleavage site for the signal

peptide. The original hypothesis of the mechanism by which pancreatitis is initiated by this mutation focused on a defect in secretion (Witt, Luck et al. 1999), but recently chymotrypsin C was shown to activate the A16V mutant four times faster than wild type cationic trypsinogen (Nemoda and Sahin-Toth 2006).

The A16V mutation was identified during a study to determine the spectrum and frequency of mutations in the PRSS1 gene in 44 children/adolescents with chronic pancreatitis (Witt, Luck et al. 1999). Thirty of these individuals were found to have idiopathic pancreatitis and fourteen to have hereditary pancreatitis. R122H was identified in one individual. A16V was found in three individuals with presumed idiopathic pancreatitis and in one said to have hereditary pancreatitis (Witt, Luck et al. 1999). The A16V mutation was also identified in seven first-degree relatives of these patients but only one had clinically apparent pancreatitis, suggesting low penetrance of this mutation (Witt, Luck et al. 1999).

1.5.5 Variants

In addition to these three principle mutations, there are multiple variants of the PRSS1 gene and these include: -28delTCC (a three base pair deletion at position base pairs upstream from the start codon)(Ferec, Raguenes et al. 1999), D19A (Chen, Kukor et al. 2003), D22G (Teich, Ockenga et al. 2000), K23R (Ferec, Raguenes et al. 1999), N29T (Pfutzer, Myers et al. 2002), P36R (Chen, Piepoli Bis et al. 2001), Y37X (Chen, Le Marechal et

al. 2003), V39A (Arduino, Salacone et al. 2005), E79K (Chen, Piepoli Bis et al. 2001), G83E (Chen, Piepoli Bis et al. 2001), K92N (Chen, Piepoli Bis et al. 2001), D100H (Tautermann, Ruebsamen et al. 2001), L104P (Teich, Bauer et al. 2002), R116C (Le Marechal, Bretagne et al. 2001; Teich, Bauer et al. 2002), R122C (Le Marechal, Bretagne et al. 2001), V123M (Chen, Piepoli Bis et al. 2001), T137M, C139S (Keiles and Kammesheidt 2006), C139F (Teich, Bauer et al. 2002), and G208A (Keiles and Kammesheidt 2006). All these variants are rare and in some cases the link with hereditary pancreatitis is only suggestive. Two neutral polymorphisms (D162D and N246N (Teich, Mossner et al. 1998)) have also been described. An updated list of PRSS1 & SPINK1 mutations is available at **http://www.uni-leipzig.de/pancreasmutation/**.

However, many families have been described with clinically defined hereditary pancreatitis where there is no PRSS1 mutation (Howes, Lerch et al. 2004; Rebours, Boutron-Ruault et al. 2009). This indicates that there may be at least one more disease gene, which remains to be identified.

1.5.6 Mechanisms of mutation induced pancreatitis

The discovery of multiple mutations in the cationic trypsinogen gene raised the intriguing question as to how they were all associated with a 'gain of function' rather than the usual finding of mutations leading to 'loss of function'. Whitcomb *et al,* with the first report of a mutation in cationic trypsinogen, proposed a mechanism via which the R122H mutation could

increase the propensity of developing acute pancreatitis (Whitcomb, Gorry et al. 1996). As further mutations were identified several hypotheses were proposed to explain the role of each mutation and how they related to a central pathogenic pathway causing pancreatitis. The five common theories of trypsinogen mutations in causing hereditary pancreatitis were (i) increased trypsin stability, (ii) increased trypsinogen autoactivation, (iii) increased susceptibility to cathepsin B mediated trypsinogen activation, (iv) loss of trypsin function, (v) chronic SPINK1 depletion.

Whitcomb proposed that the Arg122-Val123 autolytic peptide bond was an important intraacinar failsafe mechanism and the R122H mutation would result in a 'supertrypsin', that was resistant to autolytic degradation within the cell, eventually leading to pancreatitis (Whitcomb, Gorry et al. 1996).The importance of Arg122 for the autolysis of trypsin was confirmed by biochemical analysis (Sahin-Toth, Graf et al. 1999; Sahin-Toth and Toth 2000) but detailed experiments demonstrated that autolysis of cationic trypsin proceeded very slowly *in vitro* (Kukor, Toth et al. 2003). Also, the R122H mutation not only increased the stability of trypsin but also of the zymogen (Sahin-Toth, Graf et al. 1999) and increased autoactivation (Sahin-Toth and Toth 2000).

The debate has been further complicated by chymotrypsin C and its ability to degrade all three human trypsins (Szmola and Sahin-Toth 2007). In condition of low calcium concentration, chymotrypsin C selectively

cleaves the Leu81-Glu82 peptide bond within the calcium-binding loop (Szmola and Sahin-Toth 2007). This is followed by autolytic cleavage by a second trypsin molecule cleaving the Arg122-Val123 peptide bond and subsequently rapid degradation of the protein (Szmola and Sahin-Toth 2007). Millimolar calcium concentrations inhibit chymotrypsin C and protect against trypsin degradation and promote autoactivation by cleavage of the Phe18-Asp19 peptide bond (Nemoda and Sahin-Toth 2006). The R122H mutation, in this context, protects trypsin from undergoing degradation and may contribute to its pathomechanism for causing pancreatitis (Szmola and Sahin-Toth 2007).

This raised the possibility that increased trypsin stability was not responsible for hereditary pancreatitis. Work to characterise the other PRSS1 mutations found that they did not affect autolysis, thus suggesting that trypsin stability was not a common pathomechanism in hereditary pancreatitis.

The mechanism accounting for how N29I causes pancreatitis is uncertain, although in light of the assumed mechanism of action of R122H and the clinical similarities between R122H and N29I phenotypes, it was originally suggested that the mechanism must involve increased trypsin activity (Gorry, Gabbaizedeh et al. 1997) by reducing the availability of the autolysis loop and thus making the trypsin molecule resistant to degradation. Whitcomb et al predicted that the N29I mutation may induce

early trypsin activation (Whitcomb 2001), or conformational changes based on the crystallographic structure of trypsin (Whitcomb 1999) that could explain a reduced accessibility to the calcium binding pocket. An alternative model was proposed that suggested the N29I mutation alters the native structure of cationic trypsinogen to a sheet structure (Nishimori, Kamakura et al. 1999). It was implied that this conformational alteration might impair trypsin activation (Nishimori, Kamakura et al. 1999).

Biochemical approaches to investigate the mechanism concluded that the N29I mutation increased autoactivation under acidic conditions (Sahin-Toth and Toth 2000; Szilagyi, Kenesi et al. 2001), with no alteration in trypsin or zymogen stability. As increased autoactivation was seen with R122H, N29I and N29T mutation and N29I had not shown evidence of increased stability of trypsin, enhanced autoactivation became the leading hypothesis for mutant cationic trypsinogen associated pancreatitis. Finding of enhanced autoactivation in mutations D19A, D22G, K23R, affecting the trypsinogen activation peptide, strengthened the hypothesis (Teich, Ockenga et al. 2000; Nemoda and Sahin-Toth 2005). These findings are important as these mutations do not affect trypsin structure or function and can only exert their effect by altering the properties of cationic trypsinogen. This is the most widely accepted mechanism at the time of writing (Sahin-Toth 2006).

Another hypothesis suggested that pancreatitis-associated mutations may be more susceptible to activation by cathepsin B (Szilagyi, Kenesi et al. 2001), with some evidence demonstrating that the N29I mutant is activated faster by cathepsin B (Szilagyi, Kenesi et al. 2001). However, this finding was not confirmed by a later study, which found that there was no difference in activation by cathepsin B of the R122H, N29I or N29T mutants compared to wild type trypsinogen (Kukor, Mayerle et al. 2002). Work assessing the role of cathepsin B in the context of D22G and K23R mutants demonstrated that trypsinogen activation was decreased (Teich, Bodeker et al. 2002).

Some investigators found the large number of 'gain of function' mutations associated with hereditary pancreatitis to be unusual and speculated whether trypsin somehow had a protective role in pancreatitis. This could be demonstrated if the mutations caused a 'loss of function'. The discovery of R122C seemed to agree with this hypothesis (Le Marechal, Chen et al. 2001; Simon, Weiss et al. 2002). The loss of function resulted from misfolding of trypsin due to the presence of an unpaired cysteine, however, when the active trypsinogen fraction was analysed the R122C mutation had a similar profile to the R122H mutation (Simon, Weiss et al. 2002). A second mutation E79K was also shown to have decreased autoactivation however it appeared to transactivate anionic trypsinogen and thus postulated to increase anionic trypsin levels in acinar cell causing

pancreatitis (Teich, Le Marechal et al. 2004). Though this theory is interesting it does not agree with the rest of the biochemical evidence in support of enhanced autoactivation.

The chronic SPINK1 depletion hypothesis is an extension of the enhanced autoactivation theory. It stipulates that the normal inhibition of prematurely activated trypsin, as it transits through the secretory pathway, by SPINK1 is overwhelmed by mutations that enhance autoactivation of cationic trypsinogen. Saturation of this protective pathway leads to free activated trypsin able to exert a pathological effect on the acinar cell leading to pancreatitis. Evidence in support of this theory comes from identification of rare severe SPINK1 mutations that cause loss of expression of the diseased allele and appear to be associated with hereditary pancreatitis (Le Marechal, Chen et al. 2004).

1.5.7 Presentation of hereditary pancreatitis

The prevalence of hereditary pancreatitis has been estimated to be 0.3/100000 inhabitants in a French national series (Rebours, Boutron-Ruault et al. 2009). The initial presentation of patients with hereditary pancreatitis is clinically identical to sporadic cases of pancreatitis, with acute episodes mimicking gallstone, alcoholic or idiopathic acute and chronic episodes similar to any other cause of chronic pancreatitis (Charnley 2003). Childhood presentations of HP are similar to idiopathic juvenile chronic pancreatitis (Charnley 2003).

The EUROPAC group found that their cohort of patients (n = 418 affected) presented with symptoms of pancreatitis at an early age, with a median onset of symptoms at 12 years with over 70% of individuals developing symptomatic pancreatitis by the age of 20 years (Howes, Lerch et al. 2004). Lowenfels et al looked at a large cohort of individuals with hereditary pancreatitis (n = 412 affected) from 16 countries and found the mean age of symptom onset to be 14.1 years with an equal sex ratio but with a slightly more common paternal inheritance pattern (57%) (Lowenfels, Maisonneuve et al. 2000), whilst Rebours et al looked at a national cohort (n=200 affected) and concluded in their group a median age of symptom onset of 10 years (Rebours, Boutron-Ruault et al. 2009).

Howes et al demonstrated that individuals with R122H mutations presented earlier at a median age of 10 years in comparison to those individuals with the N29I mutation or compared with individuals with no PRSS1 mutation, who had a median age of presentation of 14 years and 14.5 years respectively (Howes, Lerch et al. 2004). Interestingly, Rebours et al and also Keim et al, who studied 101 individuals, failed to demonstrate any significant difference in age of symptom onset between R122H and N29I mutation carriers (Keim, Bauer et al. 2001; Rebours, Boutron-Ruault et al. 2009). There appears to be a bimodal distribution for the age of onset with a peak under the age of 10 years and another at around 20 years (Keim, Bauer et al. 2001).

Amann et al provides one of the few studies looking at identical twins in hereditary pancreatitis (Amann, Gates et al. 2001). They found that the median age of symptom onset of hereditary pancreatitis in concordant twins was almost identical, with similar ages of onset seen in matched siblings and a significantly different age of symptom onset from individuals from age, sex, and mutation-matched controls (Amann, Gates et al. 2001).

The two most frequent symptoms were pancreatic pain (83%) or a bout of acute pancreatitis (69%) (Rebours, Boutron-Ruault et al. 2009).

1.5.8 Natural clinical history of hereditary pancreatitis

The EUROPAC Study group has provided the largest detailed study of hereditary pancreatitis to date with 527 individuals recruited from 14 countries of which 418 individuals from 112 families were affected (Howes, Lerch et al. 2004). There were 58 (52%) families of whom 222 individuals (53%) were characterised by R122H mutations, 24 (21%) families had N29I mutations (94 individuals (22%)) and 21 (19%) families had no PRSS1 mutation (72 patients (17%)). They demonstrated an overall median of 1.88 attacks per year, which was unrelated to the type of PRSS1 gene mutation or gender (Howes, Lerch et al. 2004). Not all of the symptomatic episodes of pancreatitis were severe enough to warrant hospital admission, with the median number of admissions to hospital for complications of pancreatitis being 0.3 (Howes, Lerch et al. 2004; Howes,

Greenhalf et al. 2005). The number of hospital admissions was unaffected by gender, however, individuals with PRSS1 mutations did have a tendency for fewer hospital admissions than those with no identified causative mutation (Howes, Lerch et al. 2004). Approximately, 85% (158/176) of individuals reported that symptomatic episodes lasted no more than one week; the remaining 15% had attacks over one week. The duration of acute pancreatitis was not influenced by either gender or PRSS1 mutation status (Howes, Lerch et al. 2004).

Gorry et al reported on two large families with PRSS1 mutations and they found that 86% (24/28) of individuals in the R122H family had more than five hospital admissions in contrast to the N29I family where there were just 47% (7/15)(Gorry, Gabbaizedeh et al. 1997). In a larger study by Keim et al the clinical characteristics of 30 families with hereditary pancreatitis consisting of six families with the N29I mutation (n = 25) and 21 families with the R122H mutation (n = 76) were examined (Keim, Bauer et al. 2001). In the N29I group, 24% had no symptoms and 40% mild symptoms, whilst in the R122H group, 26% had no symptoms and 42% mild symptoms, though they concede that their sample size is small (Keim, Bauer et al. 2001).

The incidence of exocrine and endocrine failure in hereditary pancreatitis was shown to be high in the EUROPAC study, with the development of malabsorption in 60% at 70 years of age and a median time to

malabsorption of 53 years (Howes, Lerch et al. 2004). The cumulative risk of diabetes mellitus was 69% by 70 years of age and a median time to diabetes mellitus of 53 years. Neither exocrine nor endocrine failure was influenced by the genetic mutation (Howes, Lerch et al. 2004). Rebours et al reported exocrine and endocrine insufficiency in 34% and 26% at a median age of 29 and 38 years respectively and irrespective of mutational status (Rebours, Boutron-Ruault et al. 2009).

Surgical procedures were reported in 19% of affected individuals with 59% of those requiring pancreatic resection (Howes, Lerch et al. 2004). The time to resection was significantly reduced in women, who had a higher cumulative risk of resection in comparison to men (Howes, Lerch et al. 2004). The time to resection was also significantly reduced with the N29I mutation with an associated increased cumulative risk of resection of 35% compared to R122H (13%) and mutation negative patients (13%) (Howes, Lerch et al. 2004). The resection rate was similar in the report by Rebours et al though they did not find a difference associated with mutational status (Rebours, Boutron-Ruault et al. 2009).

1.6 Pancreatic Ductal Adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) is one of the most common causes of cancer death in the Western world (Parkin, Bray et al. 2005; Jemal, Siegel et al. 2009) with an overall 5-year survival rate of below 5% (Li, Xie et al. 2004). The incidence of PDAC approaches that of mortality

(Jemal, Siegel et al. 2009). Although only around 10% of patients are eligible for resection, prognosis is improved in patients who undergo resection followed by adjuvant chemotherapy with 5-year survival rates of up to 20-29% (Neoptolemos, Stocken et al. 2004). Certain conditions predispose patients to the development of pancreatic ductal adenocarcinoma, including increasing age, smoking (Coughlin, Calle et al. 2000), new onset diabetes mellitus (Chari, Leibson et al. 2005), chronic pancreatitis (Howes and Neoptolemos 2002), hereditary pancreatitis (Rebours, Boutron-Ruault et al. 2008) and an inherited predisposition for pancreatic cancer (Vitone, Greenhalf et al. 2006).

PDAC is believed to arise from the ductal epithelium of the pancreas (Kozuka, Nagasawa et al. 1982), although some studies suggest a possible origin from transdifferentiated acinar cells (Longnecker, Shinozuka et al. 1980). Pathological studies of PDAC point towards a multistep progression similar to the 'adenoma-carcinoma' sequence seen in colorectal cancer (Hruban, Adsay et al. 2001). The earliest change is one of squamous metaplasia, with the next lesion being described as 'pancreatic intraepithelial neoplasia' or 'PanIN' (Hruban, Adsay et al. 2001). This is subdivided into PanIN-1, PanIN-2 and PanIN-3 depending on the degree of architectural atypia present (Hruban, Adsay et al. 2001) (Fig 1.5). The malignant potential of these lesions is unknown and there is suggestion that early lesions may resolve back to normal tissue (Hernandez-Munoz,

Skoudy et al. 2008). Studies have attempted to provide a temporal description of the genetic changes associated with the progression of these lesions, implicating the role of K-Ras, p53, p16 and SMAD4, amongst others, in the development of pancreatic adenocarcinoma (Hruban and Adsay 2009) (Fig 1.5).

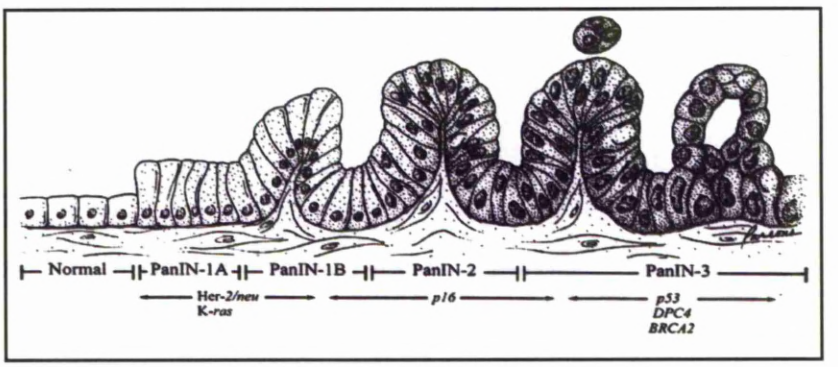

Fig 1.5 Progression model for Pancreatic adenocarcinoma

It is hypothesized that histologically normal duct cells can progress through to a severely atypical duct lesion and carcinoma in situ. (Artwork reproduced with permission from publication by Wilentz R.E et al. 2000)

1.6.1 K-Ras

K-Ras mutations have been observed in 70 – 100% of pancreatic cancers, the highest incidence of any human malignancy (Almoguera, Shibata et al. 1988). It is a G-protein involved with signal transduction and a member of the GTPase superfamily (Shields, Pruitt et al. 2000), located on chromosome 12 and encoding a 21 KDa membrane bound protein. In response to mitogenic stimuli, wild type K-Ras binds GTP and thus activates downstream signal cascades including the Raf kinases (Shields, Pruitt et al. 2000). After signalling the GTP is hydrolysed to GDP, turning off the signal cascade (Malumbres and Barbacid 2003). The commonly occurring mutations alter the proteins ability to hydrolyse GTP, effectively resulting in the K-Ras protein being persistently active and stimulating its downstream targets as though under constant mitogenic stimulus (Malumbres and Barbacid 2003). Interestingly, K-Ras mutations are present in both invasive and the earlier non-invasive cells but also in patients with chronic pancreatitis (Furuya, Kawa et al. 1997) and even seen in patients with no pancreatic disease at all (Luttges, Schlehe et al. 1999).

1.6.2 p53

Mutations in p53 are the most common mutations in human cancer, and they are seen in PDAC tissue in 27 – 76% of cases (Barton, Staddon et al. 1991; Rozenblum, Schutte et al. 1997). The p53 tumour suppressor gene is located on the short arm of chromosome 17, and encodes a transcription

factor that up-regulates genes involved in cell cycle arrest and apoptosis (Levine 1997). It is activated in response to DNA damaging stress. Mutant p53 is unable to initiate cell cycle arrest and thus to prevent tumour cell division, resulting in further accumulation of mutations in the cell (Levine, Hu et al. 2006). Most studies have not shown mutant p53 to be present in patients with chronic pancreatitis (Casey, Yamanaka et al. 1993; Apple, Hecht et al. 1999), and it is generally believed that p53 mutation occurs later than the K-Ras mutation (Hruban and Adsay 2009).

1.6.3 $p16^{INK4A}$

The $p16^{INK4A}$ gene is a tumour suppressor gene found on the short arm of chromosome 9, which inhibits the cyclin dependant kinase 4 / cyclin D1 mediated phosphorylation of the retinoblastoma protein thus inducing G_1 cell arrest (Sharpless 2005). The incidence of $p16^{INK4A}$ mutation in PDAC is around 85% (Caldas, Hahn et al. 1994) and microdissection of preneoplastic lesions has shown $p16^{INK4A}$ mutations were always found with mutant K-Ras, whereas normal pancreatic duct cells never had any $p16^{INK4A}$ mutations (Moskaluk, Hruban et al. 1997). Thus, it is assumed that the acquisition of $p16^{INK4A}$ occurs later than K-Ras but earlier than p53 in PDAC (Hernandez-Munoz, Skoudy et al. 2008).

1.6.4 SMAD4 (DPC4)

SMAD4 is homozygously deleted in around 30% of PDAC and in cases of loss of heterzygosity of one SMAD4 allele, there is a 20% rate of mutation

in the other allele (Schutte, Hruban et al. 1996). This is the highest incidence in any human cancer and reflects the importance of this gene in PDAC (Schutte, Hruban et al. 1996). SMAD4 (or Deleted in Pancreatic Cancer, DPC4) is involved in signal transduction induced by the transforming growth factor-β (TGF-β) pathway. TGF-β is a potent inhibitor of epithelial cell growth and survival (Hahn, Schutte et al. 1996).

1.6.5 Hereditary pancreatitis and pancreatic cancer risk

Lowenfels *et al* on behalf of the International Hereditary Pancreatitis Study Group estimated that the cumulative lifetime risk (to the age of 70 years) of pancreatic adenocarcinoma to be approaching 40% in patients with hereditary pancreatitis. The standardised incidence ratio (SIR), which is the ratio of observed pancreatic cancer cases in the cohort to the expected pancreatic cancers in the background population, adjusted for age, sex, and country, was 53 (Lowenfels, Maisonneuve et al. 1997). This was supported by the EUROPAC study which reported a cumulative lifetime risk of 44% to the age of 70 years and a SIR of 67 (Howes, Lerch et al. 2004) whilst Rebours et al reported a higher cumulative lifetime risk of 54% to the age of 75 and a SIR of 87 (Rebours, Boutron-Ruault et al. 2008). Lowenfels *et al* also reported that paternal transmission of hereditary pancreatitis was associated with a much greater lifetime risk of developing pancreatic cancer (Lowenfels, Maisonneuve et al. 1997) but the EUROPAC study group and the French study showed that there was no

significant difference between paternal and maternal transmission (Howes, Lerch et al. 2004; Rebours, Boutron-Ruault et al. 2008). Cigarette smoking is implicated as a risk factor related to a significantly earlier diagnosis of cancer (Lowenfels, Maisonneuve et al. 2001; Howes, Lerch et al. 2004; Rebours, Boutron-Ruault et al. 2008), with diabetes mellitus having a lesser effect (Rebours, Boutron-Ruault et al. 2008)

1.7 Transgenic Technology

1.7.1 Historical Perspective

The first successful experiment to manipulate the mouse embryo was preformed at the University of California in 1966 by Teh Ping Lin, who reported that it was possible to puncture the pronuclei of the egg, inject macromolecules such as bovine gamma globulin and transplant them into recipient female mice, with the production of viable mice at term (Lin 1966). The first microinjection of recombinant DNA was reported by Gordon *et al* in 1980 describing the somatic integration of a cloned gene consisting of fragments of the herpes simplex and SV40 virus DNA (Gordon, Scangos et al. 1980). It was not long before other groups were able to confirm these findings by successfully integrating 'transgenes' into the mouse genome (Brinster, Chen et al. 1981; Costantini and Lacy 1981; Wagner, Stewart et al. 1981; Wagner, Hoppe et al. 1981). These experiments also demonstrated that the integration of the transgenes appeared to take place in a random fashion, such that it was impossible to

predict the site or number of copies that would integrate into the genome. However, this unpredictability did not completely hinder the prospects for gene expression (Brinster, Chen et al. 1981; Wagner, Stewart et al. 1981; Wagner, Hoppe et al. 1981) or the ability to pass on the transgene to offspring (Brinster, Chen et al. 1981; Gordon and Ruddle 1981). Although transgenesis provided a powerful tool, the inability to manipulate endogenous genes in a predetermined or precise manner, limited its potential in genetic manipulation of the mammalian genome (Matthaei 2007).

Embryonic stem cells (ES cells) are pluripotent cells derived from the inner cell mass of blastocysts (Evans and Kaufman 1981). ES cells were injected into a recipient mouse blastocyst, where they were shown to contribute to all of the tissues of the adult mouse including the germ line (Bradley, Evans et al. 1984). The offspring were chimeras, as a proportion of cells were of ES cell decent and the others derived from the recipient embryo. The next step of using ES cells to introduce foreign DNA into the mouse genome was achieved when integrated retroviral DNA was identified in founders and transmitted to the offspring (Robertson, Bradley et al. 1986). The introduction of a mutant endogenous gene was reported simultaneously by two groups (Hooper, Hardy et al. 1987; Kuehn, Bradley et al. 1987), both of whom were working with hypoxanthine phosphoribosyltransferase (HPRT) gene which is defective in Lesch-

Nyhan syndrome, leading to an error in purine metabolism (Torres and Puig 2007). These were the first models of human disease generated by the manipulation of ES cells, and paved the way for gene targeted exchange of an endogenous allele by a mutated copy via homologous recombination (Hooper, Hardy et al. 1987; Kuehn, Bradley et al. 1987).

It was not long before many laboratories started to publish reports of the first 'knockout' mice (Koller, Hagemann et al. 1989; Thompson, Clarke et al. 1989; Thomas and Capecchi 1990), and there have been many further reports of targeted manipulation of the mouse genome, whereby an endogenous gene has been mutated to destroy its function (knock-out) or switched to a disease associated allele (knock-in) (Matthaei 2007).

1.7.2 Site Specific DNA Recombinase

Recently, the ability to switch a specific gene on or off has been made possible by the development of conditional genetic manipulation using site specific recombination. Two members of the integrase family of site-specific recombinases are, Cre from bacteriophage P1 (Sauer and Henderson 1989) and Flp from *Saccharomyces cerevisiae* (O'Gorman, Fox et al. 1991)

Cre catalyses recombination between two 34bp recognition sites called *loxP* (locus of crossover P1). The 34bp sequence consists of an 8bp core, which determines directionality, and is flanked by 13bp of palindromic

sequence (Nagy 2000). A gene flanked by loxP sites is referred to as a floxed gene. The Cre/lox system has been developed for use in the mouse (Lakso, Sauer et al. 1992) and usually requires a mouse strain containing a targeted gene flanked by two loxP sites to be crossed with a mouse expressing Cre recombinase. Depending on the position and orientation of the loxP sites, the DNA sequence can be excised, inverted or translocated (see Fig 1.6). When the loxP sites are flanking the target sequence in the same direction, Cre excises the intervening DNA segment, with just a single loxP site remaining. If the loxP sites are inserted in opposite orientation with respect to each other, then the DNA segment is inverted, with both loxP sites remaining. To achieve translocation, the loxP sites are located on differing strands of DNA orientated in the same direction, Cre recombinase then results in the translocation of these segments (Nagy 2000). This simple system is an extremely powerful tool allowing, with the addition of the appropriate promoters, a wide variety of genetic manipulations of the mouse genome, in a tissue or cellular specific fashion (Nagy 2000).

The Flp (flippase) recombinase is a very similar system also recognising a 34bp sequence (Flp recombinase target - FRT) (O'Gorman, Fox et al. 1991). The DNA sequence flanked by two FRT sites is referred to as 'flrted'. However, Flp is not as potent a recombinase as Cre at a body temperature of 37°C and has thus been modified to produce an enhanced

Flp (Flpe) which functions more efficiently at body temperature (Buchholz, Angrand et al. 1998).

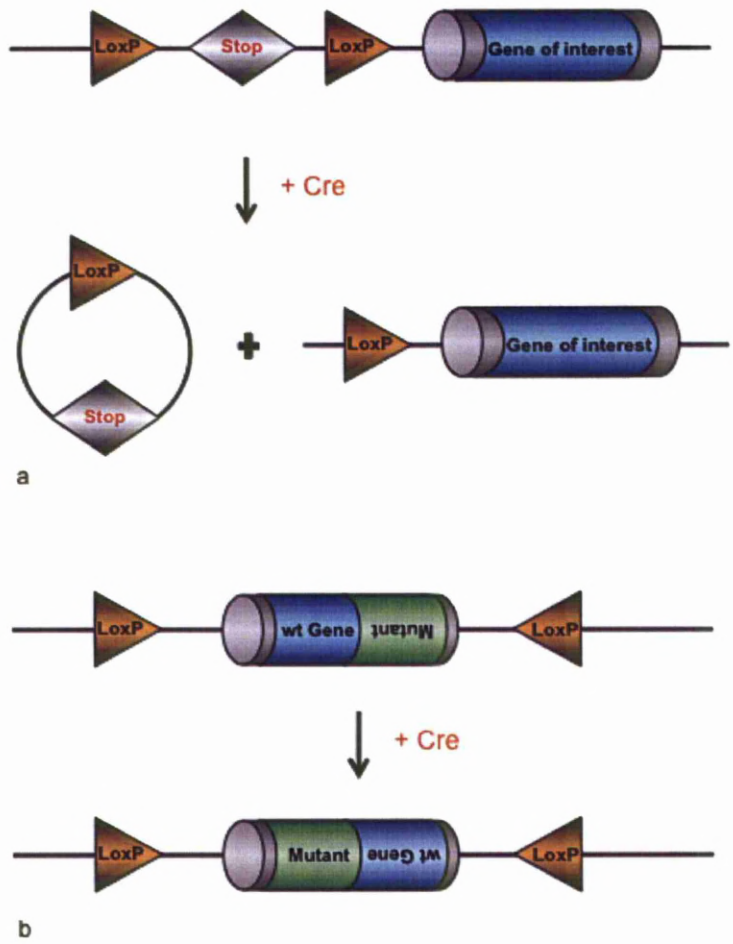

Fig 1.6 Site-specific DNA recombination

Schematic diagram showing the action of the Cre recombinase on paired *LoxP* sites with (a) excision of the DNA or gene when the *LoxP* **sites are 'in cis' and orientated the same way or (b) inversion of the** DNA or gene when the *LoxP* **sites are 'in trans' and orientated in** opposite directions.

1.7.3 The Tet-Off and Tet-On Systems

A popular tool in transgenic science is the binary transcription transactivation systems, Tet-Off & Tet-On. In the Tet-Off system gene expression is turned off in the presence of doxycycline or tetracycline, whilst in the Tet-On system gene expression is turned on in the presence of doxycycline only (Gossen and Bujard 1992).

The system is based on two components, the first a tetracycline dependant regulatory protein first identified in *E.Coli*, the Tet repressor protein (TetR)(Gossen, Freundlieb et al. 1995) that is fused to the VP16 activation domain of the Herpes simplex virus, generating a transcription activator known as tetracycline controlled transactivator (tTA) (Gossen, Freundlieb et al. 1995). This is the critical component of the original Tet-Off system, whilst the Tet-On system was created by introducing a four amino acid change in TetR resulting in a protein rtTA (reverse tTA) (Baron, Gossen et al. 1997).

The second component is the tetracycline response element (TRE) that controls expression of the gene of interest. In the presence of Doxycycline, rtTA bind to TRE and induces gene expression (see Fig 1.7), whilst in contrast, tTA is only able to bind TRE in the absence of doxycycline. For the purpose of mammalian work the Tet-On system was optimised and only responsive to doxycycline (Kistner, Gossen et al. 1996). The regulatory protein is referred to as optrtTA.

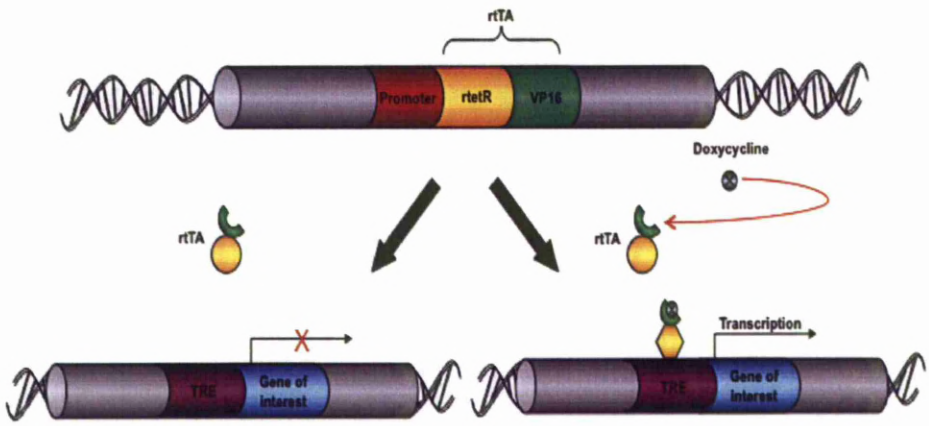

Fig 1.7 Tet-On System

Schematic diagram of the Tet-On system. The **transcription of the rtTA protein is under control of a given promoter. The rtTA protein,** in the presence of doxycycline, binds to **TRE and activates transcription** of the gene of interest.

1.7.4 Pancreas Specific Promoters

A promoter is a DNA sequence, which is recognised directly or indirectly, and bound by a DNA dependent RNA polymerase during the initiation of RNA transcription (Margaritis and Holstege 2008). The promoter lies upstream with respect to the gene of interest, and when developed into a construct for integration into the mouse genome, can confer tissue specificity and thus determine the site of transcription. The promoter may allow a gene to be widely expressed in a variety of tissues i.e. metallothionein (MT-I), actin, collagen and viral promoters (Palmiter and Brinster 1986), whilst other promoters under the influence of tissue specific transcription factors allow gene expression to be tissue specific i.e albumin promoter for the liver (Postic, Shiota et al. 1999), myogenin for skeletal muscle (Grieshammer, Lewandoski et al. 1998), lymphocyte-specific tyrosine kinase promoter for T cells (Gu, Marth et al. 1994) etc.

At the time of writing, no promoter has been identified that targets gene expression specifically to the ductal cells of the pancreas (Bardeesy and DePinho 2002; Hezel, Kimmelman et al. 2006). This obviously has implications for developing mouse models of ductal disease, in particular pancreatic adenocarcinoma. Fortunately, through an increasing understanding of pancreatic organogenesis (Kim and Hebrok 2001), other promoters have been identified that enable the development of mouse models of pancreatic cancer and pancreatitis (Hezel, Kimmelman et al.

2006). Early attempts to understand pancreatic adenocarcinoma utilised the rat elastase promoter (Ornitz, Hammer et al. 1987; Ornitz, Hammer et al. 1987; Quaife, Pinkert et al. 1987) that allows gene expression in the acinar compartment of the pancreas (Ornitz, Hammer et al. 1987). Metallothionein and Mist1 promoters also target the acinar cells though not as efficiently as the elastase promoter (Hezel, Kimmelman et al. 2006). The Pdx1 and Ptf1-p48 promoters allow expression in the common progenitors of all pancreatic cell types with relatively little expression outside the pancreas (Hingorani, Petricoin et al. 2003), and have been successfully employed to develop mouse models of pancreatic adenocarcinoma (Hingorani, Petricoin et al. 2003; Hingorani, Wang et al. 2005).

1.8 Transgenic models of pancreatitis

Genetically modified mice are a valuable tool to evaluate the *in vivo* consequences of specific genes, and their proteins, by allowing generation of gain of function (i.e. overexpression of a specific protein) or loss of function (i.e. deletion of gene) models (Matthaei 2007). These models have been extensively utilised to study the mediators of acute pancreatitis and how they cause pancreatic injury and multiple organ dysfunction syndrome (Pastor and Frossard 2001). Most models have focused their attention on the action of cytokines and chemokines and their role in the local insult and propagation of the systemic inflammatory response (Brady, Christmas et

al. 1999). Interleukin 1β (IL-1β) and tumour necrosis factor α (TNF-α) are proinflammatory mediators shown to increase in acute pancreatitis (Brady, Christmas et al. 1999). Studies using IL-1 and TNF-α receptor knockout mice demonstrated an attenuated severity of pancreatitis and improved survival (Norman, Fink et al. 1996; Denham, Yang et al. 1997) whilst mice deficient for the IL-1-converting enzyme, and therefore unable to secrete IL-1, had a reduced severity of acute pancreatitis, reduced lung injury and an increase in survival from 24%-80% (Norman, Yang et al. 1997). However, there is likely to be some redundancy in the immune response as highlighted by the study comparing acute pancreatitis induced by a choline deficient ethionine supplemented diet in either IL-1 receptor null mice or TNF-α receptor negative mice that found a similar reduction in the severity and mortality of acute pancreatitis but compound knock-out mice deficient in both receptors did not have an additive response (Denham, Yang et al. 1997). The above studies also reported that IL-6, a cytokine found to increase in proportion to the severity of the pancreatic injury, was significantly reduced in the knock-out mice (Norman, Fink et al. 1996; Denham, Yang et al. 1997). An IL-6 knock-out mouse model showed that caerulein induced pancreatitis resulted in more severe injury and a higher mortality rate compared to wild type animals (Cuzzocrea, Mazzon et al. 2002). However, plasma levels of IL-1 and TNF-α were increased, raising the possibility that deletion of IL-6 had resulted in a compensatory up-regulation of these cytokine (Cuzzocrea, Mazzon et al. 2002).

NF-κB (nuclear factor kappa light chain enhancer of activated B cells) proteins are transcription factors, which in response to a stimulus such as stress, cytokines, bacterial or viral antigens, allows rapid gene expression of proinflammatory mediators (Gilmore 2006). NF-κB is present as an inactive complex in the cytosol of cells bound to IκB (inhibitor of kappa B) and is activated by an IκB kinase (IKK) complex (Gilmore 2006). The IKK complex is composed of 2 catalytic subunits (IKK1 & IKK2) and a scaffold and regulatory component called NF-κB essential modulator (NEMO/IKKγ). Both IKK1 &IKK2 can phosphorylate IκB proteins, releasing NF-κB to enter the nucleus and induce transcription of its target genes including TNF-α, IL-1, IL-6 and ICAM1 (Gilmore 2006). To investigate the role of the NF-κB pathway in acute pancreatitis Baumann et al. developed transgenic mice expressing rtTA under the control of a rat elastase promoter to mediate acinar cell expression of IKK2 alleles (Baumann, Wagner et al. 2007). They reported that dominant negative IKK2 expression ameliorated caerulein-induced pancreatitis though there was no effect on trypsin activation (Baumann, Wagner et al. 2007). Expression of constitutively active IKK2 was sufficient to induce a phenotype of acute pancreatitis with evidence of oedema, mononuclear cell infiltration, tissue necrosis and fibrosis, again without evidence of trypsin activation (Baumann, Wagner et al. 2007).

Though these, and other models, assessing the role of cytokine in acute pancreatitis continue to increase our understanding of the disease, they are not thought to be responsible for the earliest events leading to the onset of the disease (Halangk and Lerch 2005). The current dogma in acute pancreatitis is based on inappropriate intracellular activation of pancreatic zymogens leading to autodigestion (Whitcomb 2001). Cationic trypsinogen is considered to be a key early player that is prematurely activated to cationic trypsin, which then activates other proteases leading to cellular injury (Whitcomb 2001). This hypothesis was strengthened by the finding of mutations of cationic trypsinogen in patients with hereditary pancreatitis (Whitcomb, Gorry et al. 1996). To investigate the proposal that co-localisation of cathepsin B is responsible for the premature activation of trypsinogen Halangk et al. reported a knockout mouse model of cathepsin B deficient animals with significantly reduced trypsin activity after secretagogue induced pancreatitis, however, total TAP levels were only reduced by 50% and the level of pancreatitis and systemic inflammatory response were not drastically altered (Halangk, Lerch et al. 2000). This suggested that there must be at least one other mechanism of trypsinogen activation (Halangk, Kruger et al. 2002).

The effects of trypsin inhibition were tested in a model in which rat pancreatic secretory trypsin inhibitor I, driven by the rat elastase I promoter, was over-expressed by 190% in mice acinar cells (Nathan,

Romac et al. 2005). Caerulin-induced pancreatitis was significantly ameliorated in the transgenic animals with no difference in TAP levels but trypsin activity was reduced (Nathan, Romac et al. 2005). A *Spink3*$^{-/-}$ model, in which the mouse homolog of *SPINK1* was knocked out, resulted in autophagic loss of acinar cells and death by 14.5 days after birth (Ohmuraya, Hirota et al. 2005). Trypsin activity was detected in *Spink3* deficient animals but not in wild-type controls (Ohmuraya, Hirota et al. 2006). These studies concur with previous work suggesting that trypsin activity is tightly regulated in the acinar cells and that SPINK1 is protective.

The *Spink3*$^{-/-}$ mouse model failed to address the issue that loss of function mutations in SPINK1 result in chronic pancreatitis (Ohmuraya, Hirota et al. 2005). This was also true of a study examining *CFTR* deficient mice that found only mild histological abnormalities consistent with early human cystic fibrosis but no overt signs of chronic pancreatitis (Durie, Kent et al. 2004). Other models have described various changes similar to human chronic pancreatitis, though they are not based on genetic alteration that have been described in humans, and include overexpression of keratin 8 (Casanova, Bravo et al. 1999), defective ciliary function (Cano, Sekine et al. 2006), inactivating transforming growth factor-β (TGF-β) signalling (Bottinger, Jakubczak et al. 1997), overexpression of IL-1β (Marrache, Tu

et al. 2008), deficient for protein kinase PERK (Harding, Zeng et al. 2001), and E2F1/E2F2 double mutant mice (Iglesias, Murga et al. 2004).

Hereditary pancreatitis has clinical features of acute pancreatitis leading to chronic pancreatitis and a significantly increased risk of pancreatic adenocarcinoma, and thus a faithful transgenic mouse model may allow interrogation of the pathway leading to these diseases as well as exploring the *in vivo* role of cationic trypsinogen (Ulrich, Kopras et al. 2000). Selig et al. attempted to generate a model of hereditary pancreatitis by expressing the human cationic trypsinogen with the R122H mutation targeted to the acinar cells of the pancreas by the rat elastase promoter (Selig, Sack et al. 2006). This model failed to develop any spontaneous signs of either acute or chronic pancreatitis within 18 month, but did show a slightly more severe caerulein induced pancreatitis (Selig, Sack et al. 2006). The conclusion was that the transgene expression had been insufficient to develop a phenotype (Selig, Sack et al. 2006). Archer *et al.* developed a mouse model in which they introduced an equivalent of human *PRSS1* R122H mutation into mouse trypsinogen 4 and driving the expression in acinar cells with a rat elastase promoter (Archer, Jura et al. 2006). The transgenic animals began to display a phenotype by 7 weeks of age, with evidence of acinar cell vacuolisation and evidence of cell necrosis. By 12 weeks of age there were signs of interacinar inflammatory infiltrates and collagen deposition consistent with fibrosis was seen in 24% of animal at

4-7 months of age increasing to 40% at 1 year (Archer, Jura et al. 2006). Morphological changes at 1 year included the development of tubular complexes and increased acinar cell proliferation (Archer, Jura et al. 2006). However, the results of the paper were limited by the lack of rigorous controls. The authors did not report a control group that had transgenic expression of wt-trypsinogen 4, which would allow comparison to assess whether the pathological response was due to trypsinogen over-expression or related to the mutation. There were no transgenic mice generated to ascertain whether a neutral mutation could affect a pathological response. The transgenic construct had a C-terminal FLAG epitope tag and tandem Ires-GFP element to facilitate assessment of transgene expression, however, no mechanism was developed to exclude these affecting the tertiary structure of mouse trypsinogen 4 (Archer, Jura et al. 2006). The transgene expression was confirmed in the acinar cells by immunohistochemistry, but its subcellular location was not investigated in detail to confirm whether transgenic trypsinogen 4 entered the secretory pathway and was appropriately secreted by the acinar cells (Archer, Jura et al. 2006). These shortcomings limit the conclusions that can be drawn regarding the R122H mutation in the model and raises doubts about any parallels with the human disease.

Two models have attempted to demonstrate the link between chronic pancreatitis and pancreatic adenocarcinoma (Guerra, Schuhmacher et al.

2007; Marrache, Tu et al. 2008). Marrache et al. described a IL-1β overexpressing mouse with features of chronic pancreatitis crossed with a $p53^{R172H}$ mutant mouse, and found only one mouse developed pancreatic adenocarcinoma which may have simply been due to the p53 mutation (Marrache, Tu et al. 2008). Guerra et al. in an elegant model generated mice based on a Cre/lox and Tet-On system to conditionally express mutant K-ras^{G12V} in the acinar cells of the pancreas (Guerra, Schuhmacher et al. 2007). They found that adult acinar cells were resistant to mutant K-ras mediated transformation and showed no evidence of acinar to ductal metaplasia or PanIN lesions (Guerra, Schuhmacher et al. 2007). However, in transgenic animals with caerulein-induced chronic pancreatitis PanIN lesion were seen with a third of the cohort developing invasive pancreatic cancer (Guerra, Schuhmacher et al. 2007). The pathway involved in this progression from inflammation to cancer remains to be elucidated (Guerra, Schuhmacher et al. 2007).

1.9 Aims of the Study

The aim of the study was to develop a transgenic mouse model of hereditary pancreatitis by expressing human *PRSS1* in the acinar cells of the mouse pancreas, which would allow interrogation of the *in vivo* effects of human cationic trypsinogen expression and its role into the pathways leading to acute and chronic pancreatitis and pancreatic adenocarcinoma. The transgenic mice were designed to express either wild type PRSS1 or one of the two most frequent mutations described in patients with hereditary pancreatitis; PRSS1 R122H or PRSS1 N29I. The generation of transgenic mice expressing human *wtPRSS1* would act as a control group along with a matched cohort of non-transgenic animals. These animals would then be analysed to assess whether they develop a phenotype similar to hereditary pancreatitis in humans.

The model should only express the gene of interest (i.e. *wtPRSS1*, PRSS1 R122H, or PRSS1 N29I) in the pancreatic acinar cells of the mouse, and the protein must be transported and excreted along the secretory pathway. The protein should be identifiable amongst the native mouse trypsins/trypsinogens. The transgene should not cause inadvertent developmental or functional sequelae outside the pancreas.

To achieve these aims two separate strategies were developed. The first 'inducible' model would be based on the Tet-On system and allow spatial

and temporal control of the transgene. This model will require the development of two separate constructs. The first construct, based on the pBI-G bidirectional vector, will carry the gene of interest i.e. *wtPRSS1* or one of the two mutants (termed pBI-G wtPRSS1HA, pBI-G PRSS1HA R122H, or pBI-G PRSS1HA N29I) driven by a minimal CMV promoter that also contains a TRE. The second construct, which expresses the regulator protein OptrtTA, would be driven by an upstream rat elastase 1 promoter (See Fig 1.8). Cross breeding of mice transgenic for these constructs would provide a compound mouse strain that could be induced to express the gene of interest in the acinar cells of the pancreas upon feeding with doxycycline (See fig 1.8). The second 'non-inducible' model would utilise a construct where the rat elastase promoter drives the gene of interest in the acinar cells of the mouse pancreas in an autonomous fashion. This strategy was to be a backup in the event of difficulties with the inducible model. Both models would have a Haemaglutinin (HA) tag at the C-terminus of the protein of interest to aid identification.

A table outlining the constructs described above is shown in figure 1.9.

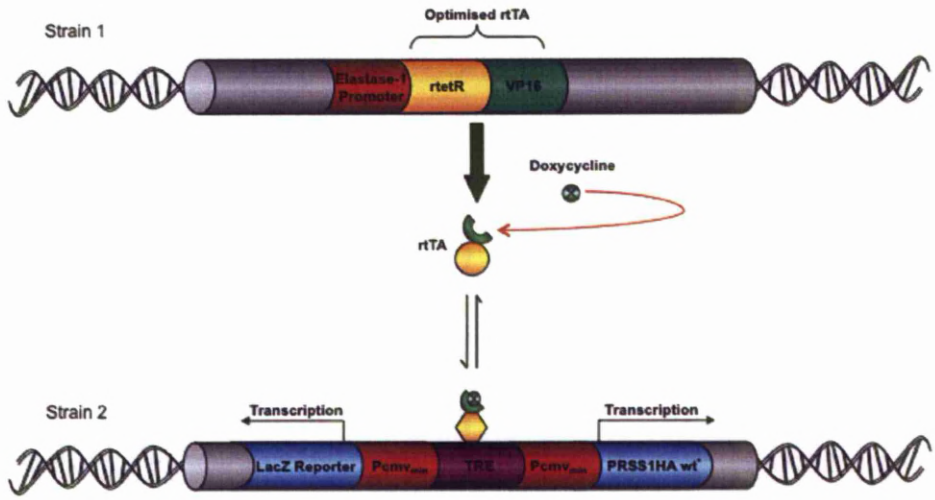

Fig 1.8 Compound transgenic mice

Schematic diagram showing the two panels of mice. Strain 1 carries the regulatory protein, OptrtTA, and strain 2 carries the gene of interest and a reporter driven by a bidirectional promoter. Cross breeding the two strains creates the compound mouse, which will express the gene of interest on feeding with doxycycline. (*The gene of interest shown is wtPRSS1HA but may also be PRSS1HA R122H or PRSS1HA N29I).

	Constructs
Non-inducible model	pBEG wtPRSS1HA
	pBEG PRSS1HA R122H
	pBEG PRSS1HA N29I
Inducible model	pBI-G wtPRSS1HA
	pBI-G PRSS1HA R122H
	pBI-G PRSS1HA N29I
	pBEG optrtTA

Fig 1.9 Constructs for non-inducible & inducible models

Table showing the constructs required to generate transgenic founder animals to develop a non-inducible and inducible mouse model of hereditary pancreatitis

Therefore the objectives of this study are:

1. To develop a set of constructs required to generate the two different models.

2. To generate potential transgenic founders for each strain by microinjection of the constructs into mouse embryos.

3. To identify transgenic animals and expand each strain. Cross breed the two panels required for the inducible model creating a compound strain for wtPRSS1HA, PRSS1HA R122H, and PRSS1HA N29I.

4. To analyse the transgenic mice to assess whether they exhibit a phenotype similar to hereditary pancreatitis.

As a secondary objective to the study, which would be undertaken concurrently with development of the transgenic animals, the *wtPRSS1* and the two mutants would be subcloned into a pCEP expression vector. These constructs would be used to transiently transfect commercially available mammalian cell lines and used to assess the reaction kinetics of cationic trypsinogen compared to the mutant proenzymes. In this way we hoped to ensure that the proteins were successfully transcribed, and most imperatively, functionally intact and capable of enzymatic activity *in vitro*.

Chapter 2

2 MATERIALS AND METHODS

2.1 Materials And Reagents

Unless otherwise stated all general chemicals and reagents used were obtained from either Sigma-Aldrich Company Ltd (Poole, Dorset, UK) or VWR International (Leicestershire, UK) and were of the highest grade available.

Chemical / Reagent / Material	**Supplier**
Acrylamide (Acrylogel 40%)	VWR
Ampillicin	Sigma
Antaractic Phosphatase	New England Biolabs Ltd
APS	VWR
β-mercaptoethanol	Sigma
Biomax Transcreen & Cassette	VWR
Blotting Grade Blocker Non Fat Dry Milk	Bio-Rad Laboratories Ltd
Bio-Rad Protein Assay Reagent	Bio-Rad Laboratories Ltd

BSA (powder)	Sigma
Cell scrapers	Corning Ltd
Chloroform	VWR
Deoxynucleotide Solution Mix (dNTPs)	New England Biolabs Ltd
Dextran Sulphate	GE Healthcare
DMEM	Sigma
DMSO	VWR
DNA ligase/buffer	Ambion/Applied Biosystems
DNA Polymerase I Klenow Fragment	New England Biolabs Ltd
EDTA	Sigma
EndoFree® Plasmid Purification Kit	Qiagen Ltd
Enterokinase Light Chain	New England Biolabs Ltd
Ethanol	VWR
Ethidium Bromide	Sigma
FBS	Sigma

Formaldhyde	VWR
Fuji Medical X-ray Film	Fisher Scientific
Geneclean® Turbo Kit	Q.BIO Gene
GeneSieve LE Agarose	Flowgen Bioscience
Glycerol	Sigma
Hybond & Hybond XL Membranes	GE Healthcare
Iodination Grade Human Trypsin	EMD Biosciences
Isopropanol	VWR
1kb DNA ladder	Invitrogen
L-glutamine	Sigma
Luria Bertani Broth Powder	Sigma
Megaprime DNA labelling system	GE Healthcare
Methanol	Sigma
Mini Protean III Electrophoresis System	Bio-Rad Laboratories Ltd
Mini Trans-Blot System	Bio-Rad Laboratories Ltd

One Shot TOP10 *E.coli*	Invitrogen
Orange G	VWR
pBI-G	Clontech Inc
PBS	Sigma
pCEP4	Invitrogen
pCR2.1	Invitrogen
^{32}P dCTP	GE Healthcare
Penicillin	Sigma
Phenol	VWR
Ponceau S	Sigma
Potassium Chloride	Sigma
Potassium Ferricynide	Sigma
Potassium Ferrocynide	Sigma
Prestained Protein Marker	New England Biolabs Ltd
Proteinase K	Sigma

QIAprep Spin miniprep Kit	Qiagen Ltd
QIAquick Gel Extraction kit	Qiagen Ltd
Restricton Endonucleases	New England Biolabs Ltd
RPMI 1640	Sigma
rTth DNA polymerase XL	Ambion/Applied Biosystems
Sheared Salmon Sperm DNA	Eppendorf Ltd
Seakem GTG Agarose	Cambrex
Sephradex G 50	GE Healthcare
SOC medium	Invitrogen
Streptomycin	Sigma
Submerged Gel Electrophoresis Units	Web Scientific
T4 DNA ligase	New England Biolabs Ltd
T4 ligase/buffer	Ambion/Applied Biosystems
Taq DNA polymerase	New England Biolabs Ltd
TEMED	VWR

Tissue culture flasks / dishes	Nunc
Total pancreatic RNA	Ambion/Applied Biosystems
Triton X-100	Sigma
Trypsin-EDTA	Sigma
Tween®20	GE Healthcare
Virkon	MRC
Western Lightning Chemiluminescence Reagent	Perkin Elmer Life Sciences

2.2 General Solutions

All solutions and buffers not supplied by the manufacturer were prepared in reverse osmosis (RO) treated water of 18MΩ.

Blocking Solution

5% Bio-Rad Blotting Grade Blocker in PBS Tween

Calcium Chloride

500mM $CaCl_2$ Stock solution

Denaturing Solution for Southern Blot

1.5M NaCl, 0.5M NaOH

Depurination Solution for Southern Blot

0.2N HCl

Freezing Medium

90% FBS, 10% dimethyl sulphoxide

LB Agar

2.5% w/v LB powder, 1.2% w/v agar, 100μg/ml ampicillin

Lysis Buffer

50mM Tris-HCl (pH 8), 100mM EDTA, 0.5% SDS

Neutralisation Solution for Southern Blot

1M Tris-HCl (pH7.4), 1.5M NaCl

PBS Tween

0.065M Na_2HPO_4, 0.015M NaH_2PO_4, 0.075M NaCl, 0.1% Tween® 20 (Polyoxyethylene sorbitan monolaurate)

Phenol:Chloroform:Isoamyl alcohol (PCI)

25:24:1 v/v

10X Ponceau S

2% Ponceau S (3-hydroxy-4-[2-sulpho-4-(sulpho-phenylazo)phenylazo]-2,7-naphthalene disulphonic acid), 30% trichloroacetic acid, 30% sulphosalicylic acid

Pre-hybridisation Solution for Southern Blot

1M NaCl, 10% Dextran Sulphate, 1% SDS

Proteinase K

10mg/ml Proteinase K in RO H_2O

Protein Gel Electrophoresis Buffer

25mM Tris, 250mM glycine, 0.1% SDS

Protein Loading Buffer (4X)

0.25M Tris-HCl (pH 6.8), 8% SDS, 40% glycerol, 0.04% bromophenol blue, 1% β-mercaptoethanol

Protein Transfer Buffer

25mM Tris, 192mM Glycine, 20% methanol

12% Resolving gel (10ml)

4.3ml H_2O, 3ml 40% acrylamide 2.5ml 1.5M Tris-HCl (pH 8.8), 0.1ml 10% SDS, 0.1ml 10% ammonium persulphate (APS), 8μl TEMED

10% Resolving gel (10ml)

As for 12% gel above but with 2.5ml 40% acrylamide and 4.8ml H_2O

SLIP Buffer

50mM HEPES (pH7.5), 10% Glycerol, 0.1% Triton X-100, 150mM NaCl, 0.5mg/ml BSA

20X SSC

3M NaCl, 0.3M sodium citrate pH 7

Stacking gel (10ml)

7.225ml H_2O, 1.275ml 40% acrylamide, 1.25ml 1M Tris (pH 6.8), 0.1ml 10% SDS, 0.1ml 10% APS, 10μl TEMED

50X TAE

2M Tris base, 2M glacial acetic acid, 50mM EDTA

1X TE

10mM Tris-HCl (pH8.0), 1mM EDTA

2.3 Antibodies

Mouse monoclonal antibodies against human cationic trypsin (13401,13402,13403,13404) were purchased from Source Bioscience Autogen. Anti-haemaglutinin (anti-HA) (12CA5) antibody used to detect HA-tagged protein was purchased from Roche Molecular Biochemicals. The anti-actin antibody (C-2), used as a protein loading control, and the anti-β-galactosidase antibody, to assess transfection, were purchased from Santa Cruz Biotechnology. Secondary antibodies, anti-mouse IgG Horseradish Peroxidase linked whole antibody (from sheep) and anti-rabbit Ig Horseradish Peroxidase linked whole antibody (from donkey), was purchased from GE Healthcare.

2.4 Primers

All primers were purchased from Eurofins MWG Operon (Germany).

OptrtTA_5' screen

5' AGT AAG GTG ATT AAC AGC GCA CTG 3'

OptrtTA_3'

5' TAC TCG TCA ATT CCA AGG GCA TCG 3'

5' BamHIPRSS1

5' GAG AGG ATC CCA CCA TGA ATC AAG CTC TCC TCA 3'

3' BamHIPRSS1

5' GAG AGG ATC CTT TAG CTA TTG GCA GCT ATG GT 3'

3' HABamHIPRSS1

5' GAG AGG ATC CTC AAA GAG CGT AAT CTG GAA CAT CGT ATG GGT AGC TAT TGG CAG CTA TGG TGT TC 3'

2.5 Plasmids

The plasmids utilised in this project are pCR2.1, pCEP4, and pBI-G, which are commercially available.

pCR2.1(Invitrogen) is an intermediate vector frequently used in cloning because it allows the direct ligation of PCR products into the vector without the use of restriction enzymes. The linearised vector has a single 3' deoxythymidine (T) residue which enables PCR products with a single deoxyadenosine (A) residue to ligate efficiently. This vector also contains ampicillin and kanamycin resistance genes allowing for positive selection in prokaryotic and eukaryotic cells respectively.

pCEP4 (Invitrogen) is a mammalian expression vector that utilises a cytomegalovirus (CMV) promoter/enhancer for transcription of recombinant genes inserted into the multiple cloning site. This vector also contains ampicillin and hygromycin B resistance genes allowing for positive selection in prokaryotic and eukaryotic cells respectively.

pBI-G (Clontech Inc) is an expression vector that utilises a bidirectional Tet-responsive promoter to drive the expression of a gene of interest and β-galactosidase simultaneously. The promoter contains the Tetracycline response element (TRE), which is responsive to the tTA and rtTA regulatory proteins of the Tet-On and Tet-Off systems respectively. The TRE is positioned between two minimal CMV promoters, which lack the

enhancer element, and therefore only allow transcription upon binding of the regulatory protein. Binding to the TRE by the regulatory protein enables simultaneous bidirectional transcription of a gene of interest and β-galactosidase. The vector contains the ampicillin resistance gene.

pBEG plasmid was a kind gift from Martin E. Fernandez Zapico, MD, from the Mayo Clinic, Minnesota, USA. It is based on the pBluescriptII SK+ vector backbone with the rat elastase promoter cloned into the *Hind*III/*Bam*HI site and human growth hormone sequence cloned into the *Bam*HI/*Not*I site. This results in a unique *Bam*HI restriction enzyme sites between these two sequences suitable for further cloning. The vector contains the ampicillin resistance gene (Fig 2.1).

pOptrtTA plasmid was a kind gift from Maria Valencik, PhD, from the University of Utah, USA. It is also based on the pBluescriptII SK+ vector backbone with the optimised rtTA (optrtTA) sequence cloned into the *Eco*RI site. The vector also contains the ampicillin resistance gene (Fig 2.2).

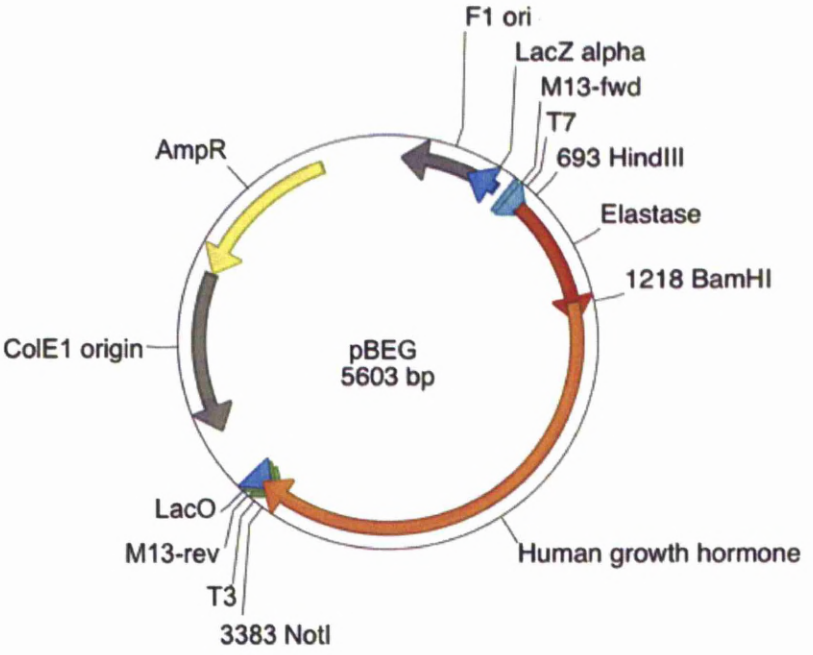

Fig 2.1 pBEG Expression Vector

Schematic diagram of the expression vector pBEG (kind gift from Dr Fernandez Zapico). Cloned into a pBluescript backbone is the rat elastase promoter and the human growth hormone polyA sequence. Unique restriction enzymes sites for *Hind*III, *Bam*HI and *Not*I are shown.

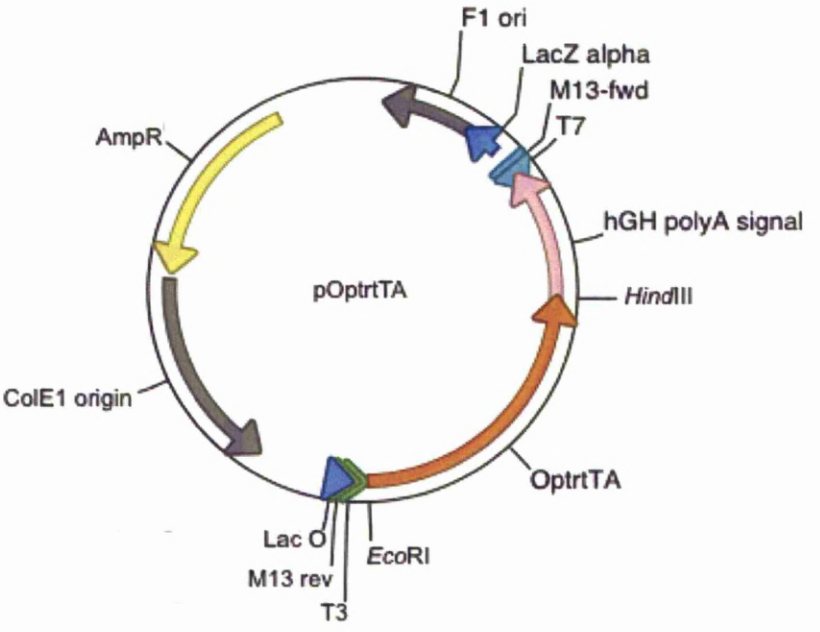

Fig 2.2 pOptrtTA Expression Vector

Schematic diagram of the expression vector pOptrtTA (kind gift from Dr Maria Valencik). Cloned into a pBluescript backbone is the OprtTA sequence and the human growth hormone polyA sequence. Unique restriction enzymes sites for *Hind*III, and *Eco*RI are shown.

2.6 Cell Culture

2.6.1 Cell Lines

Most of the cell lines used were from the cell collection held at the Division of Surgery & Oncology, University of Liverpool. Three commonly used cell lines were H1299, a human non-small cell lung carcinoma derived from a metastatic lymph node site; MCF7, a human breast adenocarcinoma cell line derived from a pleural effusion, and HEK 293T, a transformed human embryonic kidney cell line. These cell lines are also available commercially. The AR42J, a rat pancreatic acinar cell line, was a kind gift from Dr F.X Real (Unitat de Biologica Cellular i molecular, Institut Municipal d'ivestigacion Medica, Barcelona, Spain).

2.6.2 Growth Conditions

All cells were cultured as a monolayer using standard tissue culture techniques. Cells were grown at 37°C in a 95% air, 5% CO_2 humidified cell incubator. H1299 cells were maintained in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) whilst MCF7, HEK 293T were maintained in DMEM supplemented with 10% FBS and 2mM L-glutamine, and AR42J cell lines were maintained in DMEM supplemented with 20% FBS and 2mM L-glutamine.

H1299, HEK 293T and MCF7 cell lines were split when they became approximately 100% confluent whilst the AR42J cells were split when they

became ~70% confluent and patches of cells started forming domes. The medium was removed and the cells (except HEK 293T) were washed in warm PBS followed by incubation with 0.25% Trypsin-EDTA solution at 37°C for 2-4 minutes. Cells were observed under the microscope to ensure the cell layer had dispersed and then the appropriate growth medium added. The H1299, HEK 293T and MCF7 cells were split 1:10 twice a week, whilst the AR42J cells were split 1:5 twice a week.

2.6.3 Cell Seeding for Experimental Use

Cells were split from the routine growing flasks and seeded into appropriate tissue culture dishes at least 24 hours prior to use, at a confluence dependant on experimental conditions. For the purposes of transfection, cells were seeded with the aim of achieving 60-70% confluence after 24 hours.

2.6.4 Cryostorage of Cell Stocks

All cell lines were expanded and multiple aliquots frozen for storage. Confluent T175 flasks were harvested and the cells pelleted at 300xg for 4 minutes at room temperature. The pellets were re-suspended in freezing medium (90% v/v FBS, 10% v/v DMSO). The aliquots were frozen at -80°C overnight and then transferred to liquid nitrogen for long-term storage.

2.7 Transfection of Cell Lines

2.7.1 Transfection of H1299, MCF7 & HEK 293T

The day prior to transfection, cells were seeded in the appropriate culture medium to achieve a 60-80% confluence after 24hrs incubation at 37°C (5% CO_2). Transfections were performed using GeneJuice as recommended by manufacturer's instructions. Briefly, for a 100mm dish, 100μl serum free medium was mixed thoroughly with GeneJuice (3μl for every 1μg of DNA) and incubated at room temperature for 5 minutes. Typically,10μg of plasmid DNA was mixed with 2.5μg of a reporter (β-Galactosidase) plasmid DNA and then added to the GeneJuice/serum free medium solution and incubated at room temperature for 15 minutes. The GeneJuice /DNA mixture was added drop-wise to the cells in growth medium to cover the whole surface, and incubated for 24 hours at 37°C (5% CO_2). The cells were harvested by washing with PBS (except the HEK 293T cell line) and then mobilized, using a cell scraper, into 10ml of PBS. The cells were transferred into 15ml tubes and centrifuged at 300xg for 5 minutes. The resulting supernatant was removed and the cell pellets were stored at -80°C.

2.7.2 β Galactosidase In Situ Assay

Transfection efficiency was assessed by detection of the expression of exogenous β-galactosidase using an in situ assay. Cells were washed with PBS then fixed with 0.5% v/v gluteraldehyde in PBS and incubated at

room temperature for 15 minutes. Cells were washed again with PBS and then incubated with 3mM potassium ferrocynide, 3mM potassium ferricynide, 1mM magnesium chloride, and 0.5mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in PBS for 6 hours at 37°C. Successfully transfected cells stained blue.

2.8 Western Blot Analysis

2.8.1 Preparation of Whole Cell Lysates

Medium was completed removed and the cells were washed with PBS and then mobilised using a cell scraper into 10ml of PBS. The cells were transferred into 15ml tubes and centrifuged at 300xg for 5 minutes. The resulting supernatant was removed and the cell pellet was either stored at -80°C or underwent protein extraction.

2.8.2 Determination of Protein Concentration

The cell pellets were lysed using SLIP buffer and then centrifuged at 16,000xg at 4°C for 10 minutes. The supernatant was removed and the protein concentration was determined using the Bradford method (Bradford 1976). Protein standards were prepared by serial dilutions of 20mg/ml of BSA in SLIP buffer. 2μl of each standard were added to 1X Bio-Rad protein assay reagent to a final volume of 1ml and the absorbance (A) measured with an Eppendorf Biophotometer using a wavelength of

595nm to generate a standard curve. Samples were then assayed in a similar fashion to determine the protein concentration.

2.8.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Cell lysates were adjusted to 2.5μg protein per μl using 2X and 1X protein sample buffer and denatured by heating to 100°C for 5min then centrifuged at 16,000xg at 4°C for 5 minutes.

0.75mm gels were cast using the Bio-Rad Mini Protean III Electrophoresis system with an upper 5% stacking gel and a lower 10-12% resolving gel depending on the size of the protein of interest. The gels were submerged in protein gel electrophoresis buffer and typically 50μg (final volume of 20μl) of protein was loaded per well alongside 15μl of pre-stained protein marker. The samples were subjected to electrophoresis at 200V.

2.8.4 Transfer of Proteins

Hybond ECL nitrocellulose membrane was cut to size and pre-soaked in SQ water for 10 minutes before being soaked (with Whatman paper and sponges) in transfer buffer at room temperature with gentle agitation. After completion of electrophoresis, the resolving gel was set up for transfer of proteins to the nitrocellulose membrane using the Bio-Rad Mini Trans-Blot system. Transfer was carried out at 100V for 60 minutes in transfer buffer with an ice block in situ to avoid over heating of the system.

2.8.5 Protein Staining

The transfer apparatus was disassembled and the membrane was stained with Ponceau S for 5 minutes to assess transfer and even loading. The membrane was cut if necessary and then completely destained in several washes of PBS Tween.

2.8.6 Immunoblotting & Chemiluminescence

Non specific protein binding sites on the membrane were blocked using 5% Bio-Rad Blotting Grade Blocker Non Fat Dry Milk in PBS Tween for a minimum of 1 hour at room temperature with gentle agitation or overnight at 4°C. The membranes were then incubated with a primary antibody diluted in PBS Tween/milk buffer (each at 3μg/ml except anti-trypsin antibodies which were diluted 1:200) for 1hr at room temperature with gentle agitation. The primary antibody solution was removed and the membrane washed with 3 changes of PBS Tween for 15 minutes each. Next the membrane was incubated with Horseradish peroxidase conjugated secondary antibody, raised against the primary antibody species source, diluted in PBS Tween/milk buffer for 1 hr at room temperature with gentle agitation. The anti-mouse antibody was diluted to a concentration of 1:2500 and the anti-rabbit antibody to a concentration of 1:5000. The secondary antibody solution was removed and the membrane washed with 3 changes of PBS Tween for 15 minutes each.

Detection of the bound antibody complex was preformed using enhanced chemiluminescence. Equal amounts of enhanced luminal reagent and oxidizing reagent were mixed and used to completely cover the surface of the membrane for 1 minute at room temperature. Excess ECL was removed and the membrane was wrapped in cling film. The membrane was exposed to Fuji medical x-ray film in a dark room and the film developed by placing it in Kodak developer for 2 minutes and then Kodak fixer for 2 minutes before washing with tap water.

2.9 Trypsin Assay

2.9.1 Assay of cell lysates

Cells were seeded and grown as described above. If required, transient transfection of a cell line was achieved using Genejuice as per manufacturer's instructions and outlined above. After incubation for 24 hours at 37°C (5% CO_2), the cells were harvested by washing with PBS and then mobilized using a cell scraper into 10ml of PBS. The cells were transferred into 15ml tubes and centrifuged at 300xg for 5 minutes. The resulting supernatant was removed and the cell pellets were placed at -80°C for 20 minutes. The pellets were lysed in SLIP buffer without addition of any protease inhibitors and the protein concentration assessed using the Bradford method as described above.

The trypsin assay was performed for cellular lysates using the synthetic chromogenic substrate BZiPAR (Rhodamine 110, bis-(N-CBZ-L-isoleucyl-L-prolyl-L-arginine amide), dihydrochloride) at 5μM. The reaction kinetics were followed at excitation wavelength of 498nm and emission wavelength of 520nm in 0.1M Tris-HCl (pH 8.0), 1mM $CaCl_2$, 2ng/ml of human enterokinase and 150μg/ml of protein lysate at 37°C using a Hitachi F2000 Spectrophotometer. Readings were taken every 10 minutes unless stated otherwise.

During optimization of the assay, untransfected cell lines routinely available in the laboratory were analysed as described above but with the addition of 20ng/ml of high-grade recombinant human trypsin.

2.9.2 Assay of Supernatant

HEK 293T cells were grown, seeded and transfected as described above. 24 hours after transfection, the growth media was removed and replaced by OptiMEM and incubated at 37°C (5% CO_2), for a further 24 hours. The OptiMEM was removed and used for the assay in place of the 0.1M Tris-HCl (pH 8.0) with the other reagents and procedures as described above unless stated otherwise. The pH of the OptiMEM was adjusted using a weak acid or alkali as required. The cells were harvested, as described above, for Western blot analysis. Supernatant used for Western blot analysis was harvested prior to addition of any reagents for the trypsin

assay and adjusted using 4X sample buffer. The remainder of the procedure was similar to Western blot analysis as described above.

2.10 Recombinant DNA Techniques

2.10.1 Transformation of *E.Coli* with plasmid DNA

Aliquots of Top 10 *E.coli* cells were thawed and 100ng of plasmid DNA, or 5μl of ligation reaction, added and placed on ice for 30 minutes. Cells were heat shocked at 42°C for 30 seconds and replaced on ice for 2 minutes. 400μl of SOC (salt optimised and carbon) medium was added to each aliquot and the cells were then incubated at 37°C for 1 hour with agitation. The cells were grown on LB agar (2.5% & 1.2% w/v) plates containing appropriate antibiotics and incubated overnight at 37°C. If blue/white selection was required then LB agar plates coated with 32μl of 5% X-Gal and 8μl of 1M IPTG were used. Colonies were picked based on blue/white selection bias, white colonies indicating that the lac Z gene is disrupted by the successful presence of the insert.

2.10.2 Small Scale Plasmid Purification

This was performed using the QIAprep Spin Miniprep Kit according to the manufacturer's instructions. Single colonies were picked from selective plates and inoculated into 5ml of LB medium containing appropriate antibiotics and grown to saturation overnight at 37°C with agitation. Bacterial cells were harvested by centrifuging at 16000 x g at room

temperature for 5 minutes. The supernatant was removed and plasmid DNA was purified from the bacterial pellet. The procedure utilised alkaline lysis of bacterial cells followed by binding of DNA onto silica in the presence of high salt and then elution using a low salt solution. Briefly, the pellet was completely suspended in 250µl of suspension buffer (Buffer P1) containing RNase A. The pellet was then lysed in 250µl of sodium hydroxide/SDS (Buffer P2). The lysates was neutralised and adjusted to a high salt binding conditions by adding 350µl of neutralising buffer (Buffer N3). The high salt allows precipitation of denatured protein, chromosomal DNA, cellular debris and SDS, whilst plasmid DNA remained in solution. The lysate was centrifuged at 16000 x g for 10 minutes. The supernatant was transferred to a QIAprep spin column and centrifuged for 1 minute. The flow through was discarded and 0.5ml of wash buffer (Buffer PB) applied to the QIAprep spin column and centrifuged for 1 minute to remove endonucleases. The flow through was discarded and 0.75ml of a second wash buffer (Buffer PE) applied to the QIAprep spin column and centrifuged for 1 minute to remove salts. The flow through was discarded and column centrifuged for a minute to ensure removal of residual wash buffer. The DNA was eluted by adding 50µl of SQ water or elution buffer (Buffer EB 10mM Tris, pH8.5).

2.10.3 Large Scale Plasmid Purification

This was performed using the Quigen Plasmid Purification Kit according to the manufacturer's instructions. Single colonies were picked from

selective plates and inoculated into 500ml of LB medium containing appropriate antibiotics and grown to saturation overnight at 37°C with agitation. The plasmid DNA was purified using either the Qiagen Plasmid Midi kit or EndoFree Plasmid Mega Kit respectively depending on quantity of DNA required. The procedure utilised alkaline lysis of bacterial cells followed by binding of plasmid DNA to QIAGEN anion-exchange resin under low salt and pH conditions. The DNA is eluted in a high salt buffer and then concentrated and desalted by isopropanol precipitation. Briefly, bacterial cells were harvested by centrifuging at 6000 x g for 15 minutes at 4°C and the supernatant removed. The pellet was completely suspended in 4ml of suspension buffer (Buffer P1) containing RNase A. The pellet was then lysed in 4ml of sodium hydroxide/SDS buffer (Buffer P2) for 5 minutes. The lysate was neutralised and adjusted to a high salt binding conditions by adding 4ml of chilled acidic potassium acetate neutralising buffer (Buffer P3) and incubated on ice for 15 minutes. The high salt allows precipitation of denatured protein, chromosomal DNA, cellular debris and SDS, whilst plasmid DNA remained in solution. The lysate was centrifuged at 20000 x g for 30 minutes at 4°C and the supernatant removed and centrifugation repeated for a further 15 minutes at 4°C, and again, the supernatant containing plasmid DNA removed. Applying 4ml of a buffer containing sodium chloride, isopropanol and Triton X-100 (Buffer QBT), equilibrated a QIAGEN-tip 100. The supernatant was applied to the QIAGEN-tip and allowed to enter the resin

by gravity. The QIAGEN-tip was then washed with 2x 10ml of a medium salt buffer (Buffer QC) to remove all remaining contaminants. The plasmid DNA was eluted using 5ml of a high salt buffer (Buffer QF) and precipitated by adding 3.5ml of isopropanol and immediately centrifuged at >15000 x g for 30 minutes at 4°C. The supernatant was removed and the DNA pellet washed with 2ml of 70% ethanol to remove residual salt, and centrifuged at >15000 x g for 10 minutes. The ethanol is removed and the pellets air dried for 10 minutes and dissolved in TE buffer (10mM Tris, pH8; 1mM EDTA).

The procedure was slightly different when Endofree QIAGEN Megaprep Kit was used in that the volume for buffers P1-P3 is 50ml. In addition, after adding buffer P3 the lysate was not incubated on ice, but poured onto a QIAfilter Mega-Giga cartridge and incubated at room temperature. The vacuum was used to enable all of the supernatant to pass through completely. The cartridge was washed with 50ml of wash buffer containing potassium acetate (Buffer FWB2), and 12.5ml of specific endotoxin removal buffer (Buffer ER) was added to the filtered lysate, mixed by inverting and incubated on ice for 30 minutes. The QIAGEN-tip 2500 was equilibrated with 35 ml of buffer QBT and the filtered lysate was allowed to enter the resin by gravity. The QIAGEN-tip was then washed with 200ml of a medium salt buffer (Buffer QC) to remove all remaining contaminants. The plasmid DNA was eluted using 35ml of a high salt buffer (Buffer QN) and precipitated by adding 24.5ml of isopropanol and

immediately centrifuged at >15000 x g for 30 minutes at 4°C. The supernatant was removed and the DNA pellet washed with 7ml of endotoxin free 70% ethanol to remove residual salt, and centrifuged at >15000 x g for 10 minutes. The ethanol was removed and the pellets air dried for 10 minutes and dissolved in endotoxin free TE buffer (10mM Tris, pH8; 1mM EDTA). To determine the yield, DNA concentration was determined by UV spectrophotometry at 260nm and 280nm. Typical $A_{260/280}$ values were 1.8 +/- 0.05.

2.10.4 Bacterial Glycerol Stocks

Glycerol stocks were made by adding 500µl of bacterial cell culture to 500µl of 50% filter sterilised glycerol and stored at -80°C.

2.10.5 Agarose Gel Electrophoresis

This technique was used for the examination of DNA from restriction enzyme digests and DNA purification. Gels were cast by dissolving agarose into 1X TAE buffer and adding 0.5µg/ml of ethidium bromide, then allowed to set. The mini, midi & maxi submerged horizontal gel electrophoresis units were used to cast gels of 50, 100 & 150mls final volume respectively. Samples were prepared by adding Orange G (10% of final volume) and loaded onto the gel. The gel was subjected to an electrical field to achieve migration of the DNA towards the positive electrode, and the electrophoresis assessed against a 1kb ladder by UV illumination and photography of the gel. High-grade (GTG) agarose was

used for applications requiring DNA purification, whilst standard agarose was used for all other applications.

2.10.6 DNA Purification from Agarose Gel

DNA was extracted and purified from selected bands run on an agarose gel using the Q.BIOgene Geneclean Turbo Kit as per manufacturer's instructions. DNA binds to silica in high concentrations of chaotropic salt and elutes when the salt concentration is low. Briefly, the gel slice was placed in a 1.5ml microcentrifuge tube with 100µl GENECLEAN® *Turbo* salt solution per 0.1g of gel. This was incubated at 55°C for 5 minutes to melt the gel. The tube was inverted to allow mixing and the DNA/salt solution transferred to a GENECLEAN® *Turbo* cartridge. This was centrifuged at <14000 x g for about 5 second to allow all the liquid to pass through the filter. The liquid was discarded. 500µl of GENECLEAN® *Turbo* Wash solution was added to the filter and again centrifuged at <14000 x g for 5 seconds. The liquid was discarded and the wash repeated. The GENECLEAN® *Turbo* cartridge was centrifuged at <14000 x g for 4 minutes to ensure all the wash solution had been expelled. The GENECLEAN® *Turbo* cartridge was inserted into a new catch tube and 20µl of GENECLEAN® *Turbo* elution solution added directly onto the GLASSMILK® -embedded membrane, which was incubated for 5 minutes at room temperature. The DNA was eluted by centrifugation at <14000 x g for 1 minute into the catch tube. The concentration of the product was

calculated by running 10% v/v on a 0.7% w/v agarose gel and comparing the resultant band intensity with the 1636bp band that contains 10% of the mass applied to the gel.

2.10.7 DNA Restriction Digests

DNA was digested using restriction endonucleases according to manufacturer's instructions. Reactions were set up with 10 units of restriction enzyme for every 1μg of DNA with 1X recommended buffer and 1X BSA (if required) at 37°C for 2 hours, unless stated otherwise. In the case of double digests, an equal number of units of each types of enzyme were used ensuring that the total volume of the enzyme mixture did not exceed 10% of the total reaction volume and an appropriate buffer was used to allow optimal function of both enzymes.

2.10.8 Dephosphorylation of Vector DNA

After linearization of a vector, prior to cloning, the 5' ends are dephosphorylated to prevent recircularization by self ligation. Reactions were set up with 5 units of Antarctic Phosphatase for every 1μg of DNA with 1X Antarctic Phosphatase Buffer at 37°C for 15 minutes. The reaction was heat inactivated at 65°C for 5 minutes.

2.10.9 DNA Ligation Reaction

The vector and insert DNA were extracted from a 1% agarose gel and purified using the Q.BIOgene Geneclean Turbo Kit. The concentration of

the vector and insert DNA were calculated by running 10% v/v on a 0.7% w/v agarose gel and comparing band intensity against the 1636bp band. Ligation reactions were set up such that there was an insert to vector ratio of 7:1, with 1X recommended ligase buffer and 5U T4 DNA ligase in a final volume of 10μl. The reaction was incubated overnight at 12°C.

2.10.10 Polymerase Chain Reaction

For a typical 50μl reaction, 1μg of DNA template was added to a mixture containing 0.1μg of 3' primer and 0.1μg of 5' primer along with 1X recommended buffer, 800μM dNTPs, 1.5mM $Mg(OAc)_2$ and 2units rTth polymerase. The PCR reaction was set up for cycles comprising 30 seconds at 94°C to allow denaturation followed by an appropriate annealing temperature for 30 seconds and finally elongation at 70°C for 90 seconds. The hot start technique was used to reduce nonspecific amplification during the initial stages of the PCR. The product of the PCR reaction was run on a 1% w/v GTG agarose gel.

2.11 Pro-nuclear Microinjection

Microinjection of the transgenes into mice embryos was performed by Dr N Vlatković (Head of Transgenic Unit). Briefly, female mice were superovulated by intraperitoneal injection of hormones, and then mated with stud males overnight. The females that had mated successfully, as judged by the presence of a plug, were sacrificed and fertilised embryos at

a single cell stage isolated and cultured. The transgene DNA, diluted to 1-2ng/ml in microinjection quality TE buffer, was injected into one of the two visible pronuclei. The microinjected embryos were incubated overnight and those that divided into two cell stage embryos were transferred into the oviduct of a pseudopregnant female.

2.12 Isolation of Genomic DNA From Mouse Tails

Genomic murine DNA was isolated using the phenol-chloroform based method. Typically, 6-10mm of a mouse tail was clipped and placed into individually labelled 1.8ml screw cap tube corresponding to the unique mouse identifying number. Each tail was later cut into 2-3mm pieces and incubated with 0.75ml of lysis buffer and 37.5μl proteinase K overnight at 55°C in order disrupt the cells and remove proteins. The next morning, 0.75ml of phenol was added to each tube and mixed for 1hr on a rocking platform at room temperature. The solution was further mixed for 5 minutes by hand then centrifuged at 16,000xg at room temperature for 5 minutes to help separate the organic and aqueous layers. The upper, DNA containing, aqueous layer was transferred into a fresh tube containing 0.75ml of phenol:chloroform:isoamyl alcohol (PCI) and mixed by hand for 5 minutes then centrifuged at 16000xg at room temperature for 5 minutes. Again, the upper aqueous layer was transferred into a fresh tube and the DNA precipitated by adding 75μl of 3M sodium acetate (pH6) and 0.75ml of 100% ethanol then centrifuging at 16000xg at room temperature for 10

minutes. The 100% ethanol was removed and the DNA pellet washed with 1ml of 70% ethanol and centrifuged at 16000xg at room temperature for 5 minutes. The 70% ethanol was removed and the DNA pellet allowed to air dry for 5-10 minutes. The pellet was dissolved in 100µl of TE containing 10µg/ml of DNAse free RNAse. DNA concentration was calculated by measuring absorbance (A) using an Eppendorf Biophotometer at a wavelength of 260nm (A_{260} of 1 ~ 50µg of DNA/ml) and the purity of each sample determined by the ratio 260nm/280nm, with an ideal value of 1.8.

2.13 Southern Blot Analysis

A particular DNA sequence or gene can be detected in a complex mixture of genomic DNA by Southern blotting (Southern 1975). Briefly, DNA fragments are electrophoresed on an agarose gel then the DNA is denatured, to separate double stranded DNA (dsDNA) into single stranded DNA (ssDNA), and transferred onto a membrane. The fragment of interest was identified by hybridisation with a radioactive or enzyme linked complementary ssDNA (Southern 1975).

2.13.1 Restriction Enzyme Digestion of Genomic Murine DNA

Typically, 10µg of murine genomic DNA was digested using a restriction enzyme chosen for its ability as a single site cutter within the integrated transgene. The restriction endonuclease *Stu* I was used for digestion when screening animals for the presence of the *PRSS1* transgene (wild or mutant

from the pB-IG or pBEG vector). The endonuclease *EcoN* I was initially selected when screening animals for the presence of the OptrtTA transgene, however, the strategy was later revised and *Stu* I also preferred for this transgene. Digests were set up in a final volume of 30μl containing 10μg of genomic DNA, 1X recommended restriction enzyme buffer and 10U of *Stu* I per μg of DNA or 15U of *EcoN* I per μg of DNA. The digests were incubated at 37°C overnight.

Samples were run on a 0.7% w/v agarose gel and the electrophoresis assessed by UV illumination and photography of the gel.

2.13.2 Single Copy Calculation

Controls equivalent to known number of copies of the transgene, typically 0.1, 1, 10 and 20, that would allow assessment of copy number of the transgene in the positive animals as well as sensitivity of the probe used in Southern Blotting, were also run on the agarose gel in addition to the samples. The transgene integrates into the pronuclear genome completely randomly, as a single unit or multiple tandem copies, usually in a head to tail fashion as a concatamer. There is a simple formula to calculate the equivalent of a single copy of a trasgene:

$$\frac{\text{Mass of transgene DNA}}{\text{N μg genomic DNA}} = \frac{\text{N bp transgene DNA}}{3 \times 10^9 \text{ bp genomic DNA}}$$

If, for instance, a transgene is 3.6kb and 10μg of mouse genomic DNA is used then the mass of the transgene is (3600bp x 10μg DNA)/3 x 10^9 bp of mouse genomic DNA. This gives a mass of ~12pg.

2.13.3 Capillary Transfer of DNA To Membrane

After completion of electrophoresis, the gel was placed in a glass dish and wash using denaturing solution (1.5M NaCl, 0.5M NaOH), for 3 x 15 minutes each at room temperature with gentle agitation. The gel was depurinated in 0.2N HCl for 5 minutes at room temperature then washed in RO H_2O. The gel was washed in neutralisation solution (1M Tris-HCl pH7.4, 1.5M NaCl) for 3 x 15 minutes at room temperature with gentle agitation. Finally the gel and Hybond XL nitrocellulose membrane (pre-soaked in RO H_2O) were rinsed in 10X SSC transfer buffer for 30 minutes.

10X SSC transfer buffer was placed in a glass dish with a glass platform positioned at its centre (see Figure 2.3). Two 3MM Whatman paper wicks (pre-soaked in 10X SSC) were placed onto the plate, ensuring the ends were submerged into the transfer buffer, and the gel placed inverted on top so that its underside is now uppermost. The top of the gel was covered with nitrocellulose membrane, cut exactly to the size of the gel, followed by 3MM Whatman paper. Paper towels were stacked, cut slightly smaller than the gel, over the 3MM Whatman paper to a height of 8cm and then a glass plate was balanced on top and weighed down with a ~500g weight.

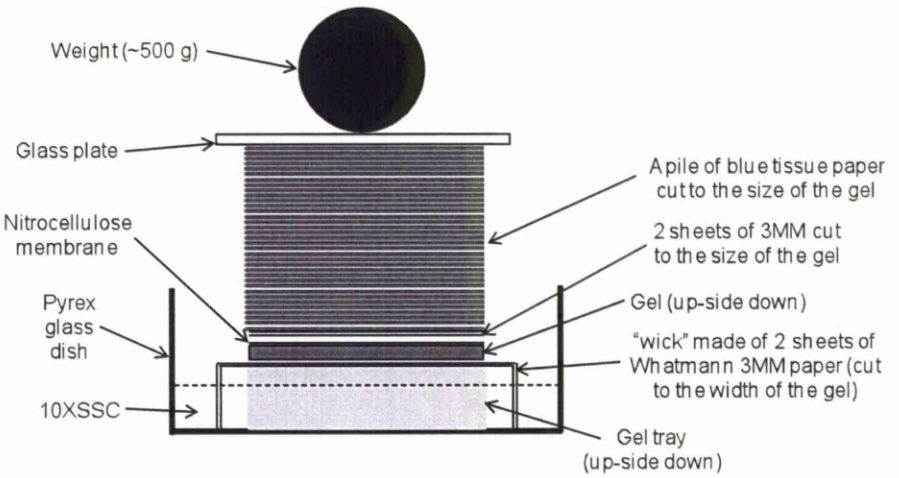

Fig 2.3 Transfer of DNA onto a membrane

Schematic diagram demonstrating a set up to allow trasfer of DNA from the agarose gel onto a nitrocellulose membrane .

The entire transfer setup was wrapped in cling-film, allowing transfer to occur overnight (Fig 2.3).

Following transfer, the membrane was air-dried, and then the DNA was fixed by cross-linking using UV irradiation at a wavelength of 254nm and 0.12J cm^2. The membrane was then soaked in 1M NaCl for a minimum of 30 minutes at room temperature with gentle agitation. Following this the membrane was placed in a hybridisation bottle with 20 ml of pre-hybridisation solution (1M NaCl, 10% Dextran Sulphate, 1% SDS) and incubated for 3-4 hours or overnight at 65°C with gentle agitation.

2.13.4 Probe Preparation

50ng of the appropriate DNA probe was labelled using the Amersham MegaPrime DNA Labelling System and 50μCi [α-^{32}P] dCTPs according to manufactures instructions. Briefly 50ng of DNA probe was incubated with 5μl of random primers and heated to 100°C for 5 minutes then slowly cooled to room temperature. 10μl of labelling buffer (containing unlabelled nucleotides), 2μl Klenow enzyme and 5μl of [α-^{32}P] dCTPs were added to a final volume of 50μl and incubated at 37°C for 45 minutes. Chilling on ice for 5 minutes stopped the reaction.

2.13.5 Probe Purification

The probe was purified by passing through a Sephredex G-50 column. Briefly, a glass pipette was plugged with a piece of siliconised glass wool

at the bottom and packed with Sephradex G-50 equilibrated in RO H_2O. 20µl of Dextran Blue (1mg/ml) and 20µl of Orange G (1mg/ml) were added to the labelled probe, which was then applied onto the column. Column was washed with RO H_2O and fractions collected. High molecular weight fraction containing the desired labelled probe was eluted with Dextran Blue.

2.13.6 Scintallation Count And Hybridisation

2µl of eluted probe was placed in a scintillation counter with a program for ^{32}P and a reading taken to calculate the volume of probe required to achieve 10cpm/ml of hybridisation solution.

The probe (at a calculated volume) was denatured by boiling for 10 minutes with Salmon Sperm DNA (100µg/ml final concentration in prehybridisation solution) and then chilled on ice for 5 minutes before mixing with 20mls of prehybridisation solution and hybridised at 65° overnight.

The following day the membrane was removed from the hybridisation bottle and washed in two changes of 2X SSC for 20 minutes at room temperature with gentle agitation. Two stringent washes were performed in 2X SSC, 0.1% SDS at 65°C for 20 minutes each with gentle agitation, followed by two washes in 0.1X SSC for 20 minutes at room temperature with gentle agitation. The membrane was finally mounted on 3MM

Whatman paper, wrapped in cling film and exposed to Kodak Biomax MS film which was placed between a Biomax TranScreen-HE intensifying screen and incubated at -80°C for 2-48 hours.

2.13.7 Film Development

The film cassette was removed from the -80°C freezer and allowed to reach room temperature. The film was removed in a dark room and placed in Kodak developer for 2 minutes and then into Kodak fixer for 2 minutes before washing in tap water.

Chapter 3

3 GENERATION OF EXPRESSION PLASMIDS

3.1 Introduction

The aim of this work was to generate genetically modified mice that stably express the wild-type human cationic trypsinogen gene, or one of the two common mutated alleles, in the acinar cells of the pancreas. To achieve this aim, we first set out to establish a set of expression plasmids carrying the genes of interest that would be utilised in generating the transgenes for microinjection.

The work to generate the cDNA of *wtPRSS1* and then introduce the point mutation, R122H or N29I, was performed by my predecessor Catherine Merriman. Briefly, the cDNA of *PRSS1* was synthesised from human pancreatic RNA by reverse transcription. The *PRSS1* cDNA was amplified by PCR using specific primers 5'PRSS1 and 3'PRSS1, and the product was cloned into the intermediate vector, pCR2.1 and transformed into XL1 Blue cells. Clones were picked and plasmid DNA purified and sent for sequencing. Once the sequence had been successfully confirmed further clones were generated using specific primers to introduce a Haemaglutinin (HA) tag, which would allow antibody mediated detection of the protein, to the 3' end of the sequence and *Not*I or *Bam*HI restriction enzymes sites at both 3' and 5' ends to enable sub-cloning into the expression vectors

pCEP, pBI-G and pBEG. The mutations, R122H and N29I, were introduced by PCR mutagenesis and the products were ligated into the intermediate vector pCR2.1. The wild-type PRSS1 and the two mutants were all generated with and without a HA tag. The sequence of each construct was confirmed.

3.2 Results

3.2.1 Generation of pCR2.1 PRSS1HA N29I

The PRSS1HA N29I mutant was cloned into the pCR2.1 intermediate vector. The PRSS1HA N29I construct was generated using pBI-G PRSS1 N29I as a template for PCR and the primers 5'BamHIPRSS1, 3' HABamHIPRSS1 (as described in section 2.10.10) in order to create *Bam*HI restriction sites necessary for subsequent cloning into the pBEG vector (see sections 3.2.3. and 3.2.4.). The sample was then subjected to agarose gel electrophoresis, the 800bp band containing PRSS1HA N29I excised and the DNA extracted. pCR2.1 and PRSS1HA N29I were then ligated to produce pCR2.1 PRSS1HA N29I (see section 2.10.9 and figure 3.1). The ligation products were transformed into One Shot® Top10 *E.Coli* competent cells (see section 2.10.1). Colonies were picked based on blue/white selection bias, white colonies indicating that the lac Z gene is disrupted by the successful presence of the insert. The plasmid was then commercially sequenced to confirm the fidelity of the insert.

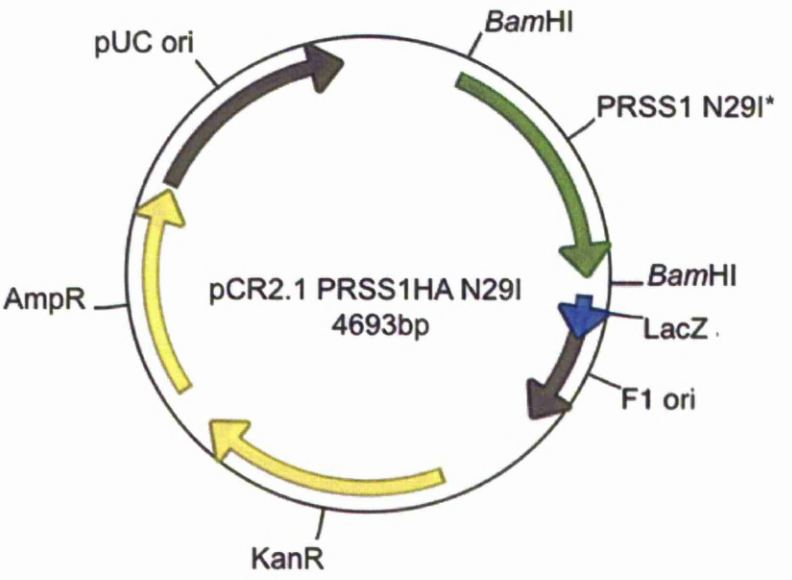

Fig 3.1 pCR2.1 PRSS1HA N29I Intermediate Vector

A schematic map of the intermediate vector pCR2.1 with the insert PRSS1HA N29I ligated to generate the plasmid pCR2.1 PRSS1HA N29I. (*shown is the N29I mutant but the vector may also represent pCR2.1 wtPRSS1HA or pCR2.1 PRSS1HA R122H)

3.2.2 Generation of pCR2.1 PRSS1 N29I

The PRSS1 N29I mutant without the HA tag was cloned into the pCR2.1 intermediate vector. These untagged constructs would not feature in the initial efforts to generate transgenic animals, but as the potential biological or biochemical effect of the HA tag could not be anticipated in our animal, these constructs could provide great utility in the future. The PRSS1 N29I construct was generated using pBI-G PRSS1 N29I as a template for PCR and the primers 5'BamHIPRSS1, 3' BamHIPRSS1 (as described in section 2.10.10). The sample was then subjected to agarose gel electrophoresis and the 800bp band containing PRSS1 N29I excised and the DNA extracted. pCR2.1 and PRSS1 N29I were then ligated to produce pCR2.1 PRSS1 N29I (see section 2.10.9 and figure 3.2). The ligation products were transformed into One Shot® Top10 *E.Coli* competent cells (see section 2.10.1). Colonies were picked based on blue/white selection bias. The plasmid was then commercially sequenced to confirm the fidelity of the insert.

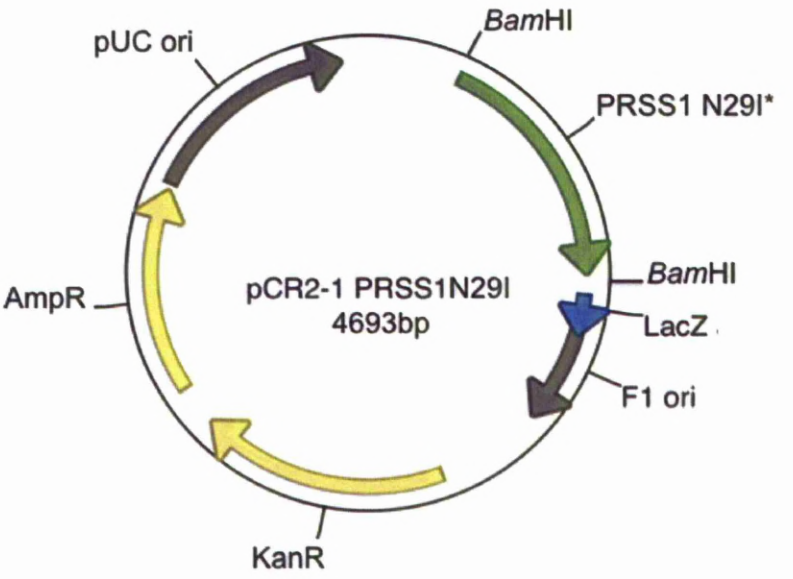

Fig 3.2 pCR2.1 PRSS1 N29I Intermediate Vector

A schematic map of the intermediate vector pCR2.1 with **the insert PRSS1 N29I ligated to generate the plasmid pCR2.1 PRSS1 N29I. (*shown is the N29I mutant but the vector may also represent pCR2.1** wtPRSS1 or pCR2.1 PRSS1 R122H)

3.2.3 Generation of pBEG PRSS1HA mutants

Plasmids were generated with the genes of interest downstream of the rat elastase I promotor, thus allowing expression to occur only in the acinar cells of the mouse pancreas.

The PRSS1HA R122H and PRSS1HA N29I sequences were excised from the intermediate vectors pCR2.1 PRSS1HA R122H and pCR2.1 PRSS1HA N29I respectively using the restriction endonuclease *Bam*HI (see section 2.10.7). Similarly, pBEG was linearised by digestion with *Bam*HI and dephosphorylated with Antarctic phosphatase to minimise self-ligation (see section 2.10.8). The samples were subjected to agarose gel electrophoresis, the bands containing pBEG and PRSS1HA R122H or PRSS1HA N29I extracted (as described in section 2.10.6). pBEG was then ligated with either PRSS1HA R122H or PRSS1HA N29I to produce pBEG PRSS1HA R122H and pBEG PRSS1HA N29I (see section 2.10.9 and figure 3.3). A restriction digest using EcoRI, which yielded fragments of 2.8kb and 3.5kb, confirmed the correct orientation.

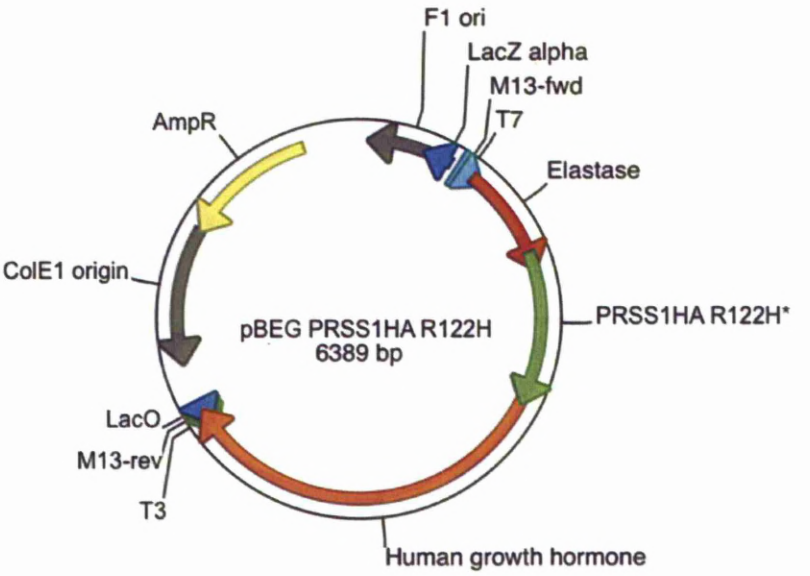

Fig 3.3 pBEG PRSS1HA R122H Vector

A schematic map of the expression vector pBEG with the **insert PRSS1HA R122H ligated to generate the plasmid pBEG PRSS1HA R122H. (*shown is the R122H mutant but the vector may also** represent pBEG wtPRSS1HA or pBEG PRSS1HA N29I)

3.2.4 Generation of pBEG PRSS1 mutants

The pBEG PRSS1 R122H and pBEG PRSS1 N29I constructs (without the HA tag) were generated and verified (see figure 3.4) in a similar manner to the pBEG PRSS1HA constructs (described in section 3.2.3).

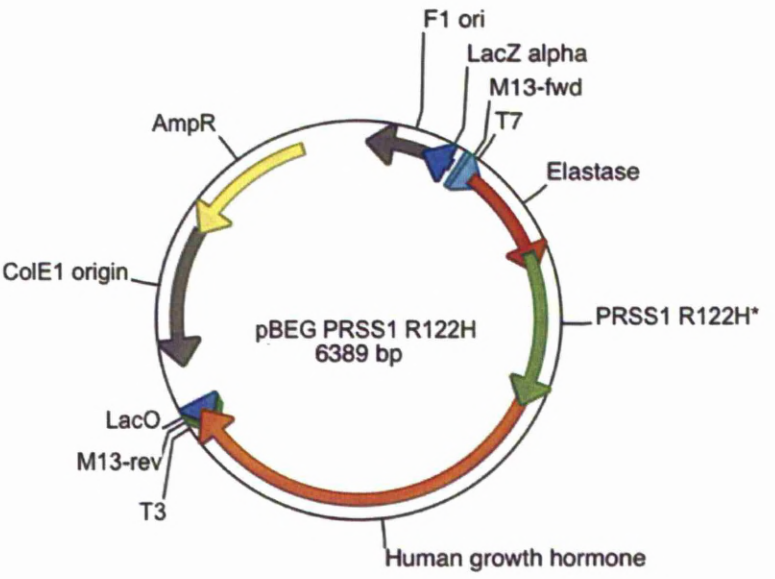

Fig 3.4 pBEG PRSS1 R122H Vector

A schematic map of the expression vector pBEG with the **insert PRSS1 R122H ligated to generate the plasmid pBEG PRSS1 R122H. (*shown is the R122H mutant but the vector may also represent pBEG wtPRSS1** or pBEG PRSS1 N29I)

3.2.5 Generation of pBEG OptrtTA

The plasmid, pOptrtTA, containing the optimised rtTA (OptrtTA) sequence was a kind gift from Dr M. Valencik, PhD. In order to ensure that expression of the OptrtTA inducer would take place specifically in the acinar cells of the mouse pancreas, the gene needs to be driven by a well-defined upstream rat elastase I promotor (see section 1.7.4). In a compound mouse, OptrtTA would bind to the Tet responsive element (TRE) in the presence of doxycycline leading to transcription and expression of our gene of interest.

To achieve this, the OptrtTA fragment was liberated from the pOprtTA vector by restriction digest with *Hind*III and *Eco*RI restriction enzymes, blunted (see section 2.10.6) and cloned into the expression vector pBEG that has been digested with the restriction enzyme *Bam*HI and treated with Antarctic Phosphatase to minimise self ligation (see section 2.10.8).

The samples were subjected to agarose gel electrophoresis and the bands containing pBEG and OptrtTA were extracted and ligated (see figure 3.5). A restriction digest using *Sac*I, which yielded fragments of 4.3kb, 1.6kb and 0.8kb, confirmed the correct orientation. The plasmid was then commercially sequenced to confirm the fidelity of the insert.

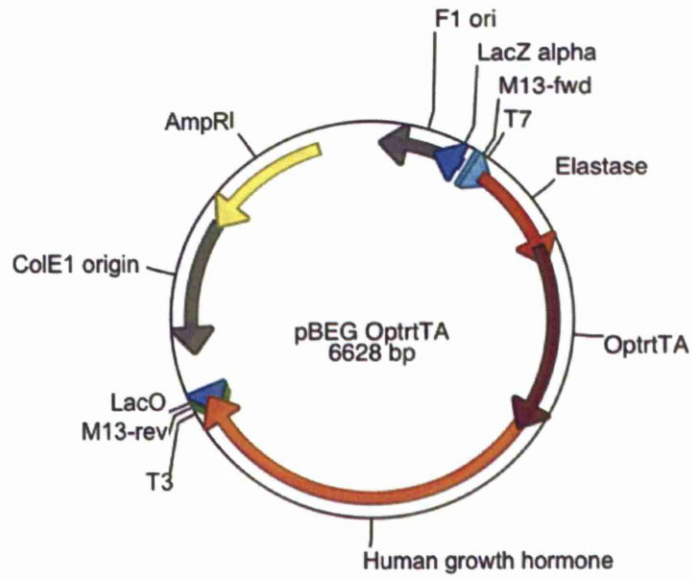

Fig 3.5 pBEG OptrtTA Vector

A schematic map of the expression vector pBEG with the insert OptrtTA ligated to generate the plasmid pBEG OptrtTA.

3.2.6 Generation of pCEP PRSS1

The plasmids described above could not be used for generic expression studies in mammalian cells in culture, since the rat elastase I promotor would be activated only in the pancreatic acinar cells. Attempts to transiently transfect AR4-2J cells, which are derived from rat acinar cells, or 266-6 cells, derived from mouse acinar cells, with any plasmid were unsuccessful due to very poor transfection efficiency. Therefore, to test the expression of the wtPRSS1 and mutants (with and without the HA tag), the genes of interest were subcloned into the pCEP vector where they would be under the control of a CMV promotor. This allowed transient transfection of human cell lines in culture, i.e H1299 or MCF7.

A set of plasmids with the *wtPRSS1* or the mutant genes (with and without the HA tag) in a pCEP backbone had already been generated in our laboratory. However, the pCEP PRSS1 vector did not express due to omission of the ATG codon and this was therefore recloned.

The *wtPRSS1* was excised from the intermediate vectors pCR2.1 PRSS1 using the restriction endonuclease *Not*I (see section 2.10.7), and subcloned into the pCEP vector that was linearised by digestion with *Not*I and dephosphorylated with Antarctic phosphatase to minimise self ligation (see section 2.10.8). The samples were subjected to agarose gel electrophoresis, the bands containing pCEP and *wtPRSS1* extracted (as described in section 2.10.6). pCEP was then ligated with *wtPRSS1* to produce pCEP wtPRSS1

(see figure 3.6). A restriction digest using *Eco*RI and *Bam*HI, which yielded fragments of 5.3kb and 2.3kb and 0.7kb, confirmed the correct orientation.

Protein expression of the pCEP-based vectors was demonstrated by Western blotting following transient transfection of H1299 cells (as described in section 2.7 and 2.8). This also confirmed that the HA tagged proteins could be detected by the monoclonal α-HA antibody and that both the tagged and untagged proteins could be detected using a monoclonal α-PRSS1 antibody (see figure 3.6).

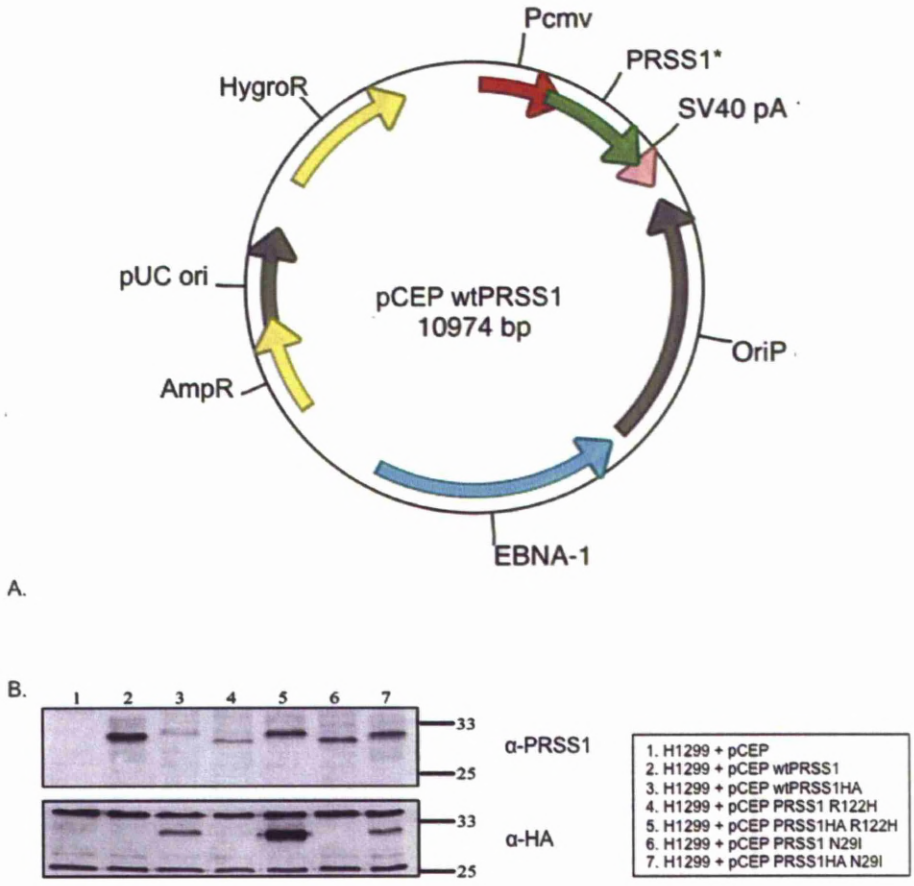

Fig 3.6 pCEP PRSS1 Vector and Western blot

(A) Schematic map of the expression vector pCEP with the insert *wtPRSS1* (*the vector may represent *wtPRSS1,* or the two mutants, with or without the HA tag) ligated to generate the plasmid pCEP wtPRSS1. (B) 50g of total protein in each lane on a 12% polyacrylamide gel according to Laemmli (1976). Protein was transferred to nitrocellulose membrane via electrotransfer and expression of the whole pCEP-based library detected (lanes 2-7) using antibodies against the HA tag or an epitope on human PRSS1.

3.2.7 pBI-G plasmid

The pBI-G based plasmids were already available in our laboratory. They had been generated using similar techniques as described above. Briefly, *wtPRSS1* and the two mutants had been generated with a C-terminus HA tag and *Not*I sites at both termini. They were cloned into the intermediate vector pCR2.1 and then subcloned into the commercially available pBI-G plasmid using the *Not*I site (see figure 3.7).

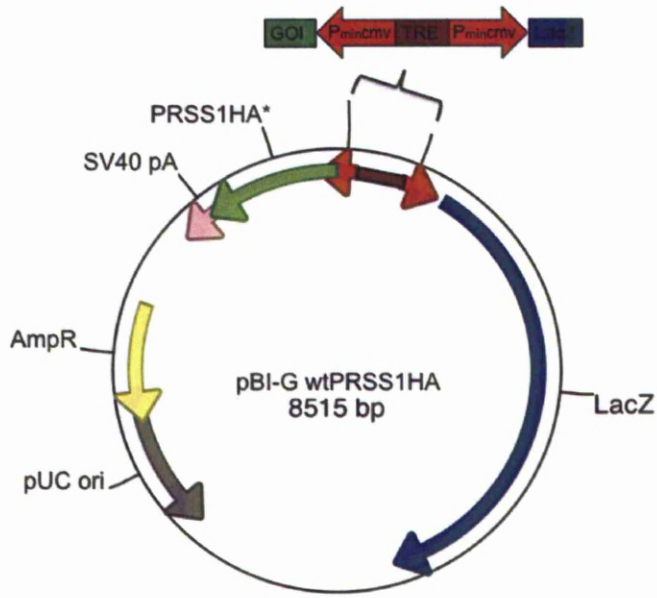

Fig 3.7 pBI-G wtPRSS1HA Vector

A schematic map of the expression vector pBI-G **wtPRSS1HA (*the** map may also represent pBI-G PRSS1HA R122H or pBI-G **PRSS1HA** N29I). The Tet responsive element (TRE) with the bi-**directional** promotors transcribing the *lacZ* **and gene of interest** (GOI), *wtPRSS1HA,* **(*may represent a map for PRSS1HA R122H** or PRSS1HAN29I) is shown in detail.

3.3 Summary

A series of vectors were created in order to generate appropriate transgenes for the development of genetically modified mice expressing human wild-type PRSS1 or one of the two common mutations. This collection of plasmids would allow development of an inducible transgenic model and a second model with constitutively expressed proteins, specifically within the acinar cells of the pancreas. In addition, plasmids were created to allow transient transfection of human cell lines, based on the pCEP backbone, utilising a CMV promotor to drive expression of the genes of interest. Protein expression of all these constructs was demonstrated by Western blotting after transfection of the plasmids into H1299 cells.

The completed set of plasmids is shown in figure 3.8 with those shown in black text already available in our laboratory whilst those in blue were created as part of this project.

pCR2.1	wtPRSS1	wtPRSS1HA
	PRSS1 R122H	PRSS1HA R122H
	PRSS1 N29I	PRSS1HA N29I
pCEP	wtPRSS1	wtPRSS1HA
	PRSS1 R122H	PRSS1HA R122H
	PRSS1 N29I	PRSS1HA N29I
pBEG	wtPRSS1	wtPRSS1HA
	PRSS1 R122H	PRSS1HA R122H
	PRSS1 N29I	PRSS1HA N29I
	OptrtTA	
pBI-G		wtPRSS1HA
		PRSS1HA R122H
		PRSS1HA N29I

Fig 3.8 Table of plasmids

A table showing all the plasmids created to allow the generation of genetically modified mice expressing our genes of interest within the acinar cells of the mouse pancreas. (Plasmids in black text were created by Catherine Merriman)

Chapter 4

4 GENERATION OF MICE WITH INDUCIBLE TRANSGENES

4.1 Introduction

Having created a set of plasmid constructs, the aim was to generate genetically modified mice with 'inducible' protein expression, based on the Tet-On system, allowing spatial and temporal control of the transgene expression. The first construct, based on the pBI-G bidirectional vector, would carry the gene of interest i.e. wt PRSS1 or one of the two mutants and the second construct, which expresses the regulator protein OptrtTA, would be driven by an upstream rat elastase 1 promoter. Cross breeding these two strains of mice would generate a compound mouse strain that could, upon feeding with doxycycline, be induced to transcribe the gene of interest in the acinar cells of the pancreas (see figure 4.1).

4.2 Results

4.2.1 Preparation of transgenes

The transgenes for pBI-G wtPRSS1HA, pBI-G PRSS1HA R122H and pBI-G PRSS1HA N29I were liberated in a similar fashion, by digesting the constructs with the restriction enzyme *Ase*I. The samples were subjected to agarose gel electrophoresis and the 7.3kb band containing the transgene was extracted (as described in section 2.10.6) (see figure 4.2).

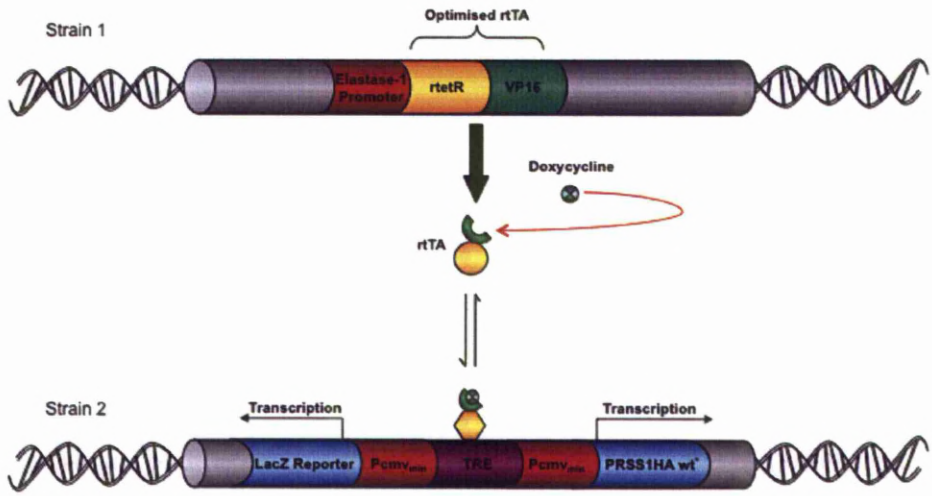

Fig 4.1 Compound transgenic mice

Schematic diagram showing the two panels of mice. Strain 1 carries the regulatory protein, OptrtTA, and strain 2 carries the gene of interest and a reporter driven by a bidirectional promoter. Cross breeding the two strains creates the compound mouse, which will express the gene of interest on feeding with doxycycline. (*The gene of interest shown is wtPRSS1HA but may also be PRSS1HA R122H or PRSS1HA N29I).

The pBEG OptrtTA construct was digested using the restriction enzymes *Hind*III and *Not*I liberating a 3.6kb band containing the transgene (see figure 4.3). The transgenes were used for pronuclear microinjection of fertilised mouse eggs (see section 2.11).

4.2.2 Preparation of Probes for Southern Blotting

In order to identify successful integration of the transgene into the murine genome, the litter from microinjected animals was screened using a Southern blot technique (see section 2.13). This required a radionucleotide labelled probe to allow specific hybridisation with a corresponding sequence of the integrated transgene.

The screening of animals microinjected with the transgenes pB-IG PRSS1HA wt, pB-IG PRSS1HA R122H or pB-IG PRSS1HA N29I was performed using the same probe. The probe was prepared by digesting the native pB-IG plasmid using the restriction endonucleases *Xba*I and *Eco*RV and *Not*I (see figure 4.2). The samples were subjected to agarose gel electrophoresis and the 1203bp band containing the probe was extracted and prepared as described in section 2.13.

The screening of the pBEG OprtTA transgene was performed using a probe prepared by PCR. Using the pBEG OptrtTA construct as a template, OptrtTA_5' screen and OptrtTA_3' screen primers were utilised to generate the probe (see section 2.10.10 and figure 4.3). The samples were

subjected to agarose gel electrophoresis and the 900bp band containing the probe was extracted and prepared as described in section 2.13.

4.2.3 Establishing Founder Animals

After microinjection of the mouse embryos, the newborn pups were screened by Southern blot analysis to identify founder animals. The mice that were found to be positive carriers of the transgene were backcrossed with wild type, non-transgenic mice. This allowed each lineage to be expanded, and also, enabled the segregation of the transgene if it had integrated into multiple sites of the murine genome. Wild type murine DNA was always digested and loaded with the samples to exclude false positive identification. Transgene DNA was loaded at known concentrations to help estimate the number of copies integrated (see section 2.13.2).

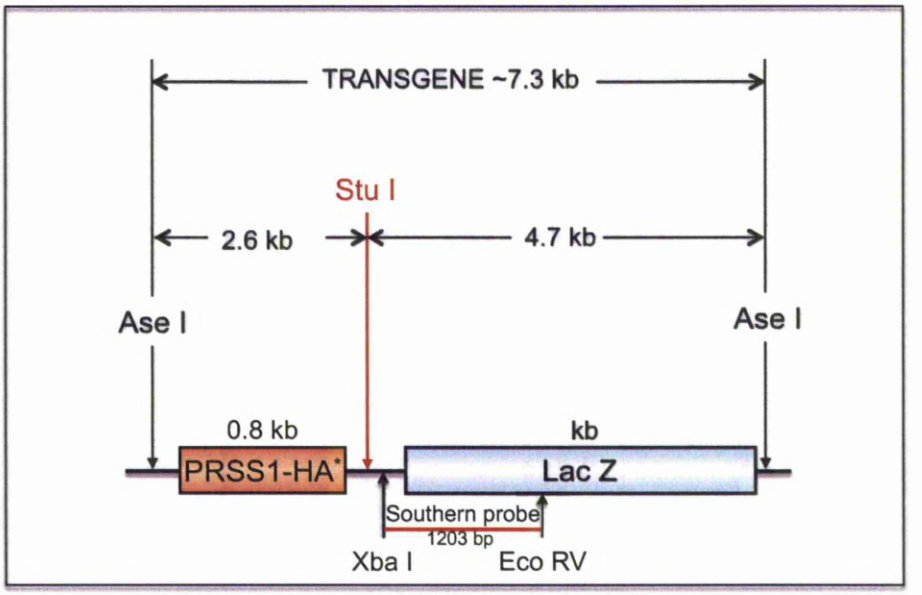

Fig 4.2 pBI-G wtPRSS1HA transgene

Schematic diagram representing the pBI-G **wtPRSS1HA transgene (*may also represent PRSS1HA R122H or PRSS1HA N29I). Shown** are the *Ase*I digestion sites used to liberate the transgene. The *Stu*I **site is cut when digesting genomic DNA and the Southern probe is** liberated by digestion with *Xba*I and *Eco*RV.

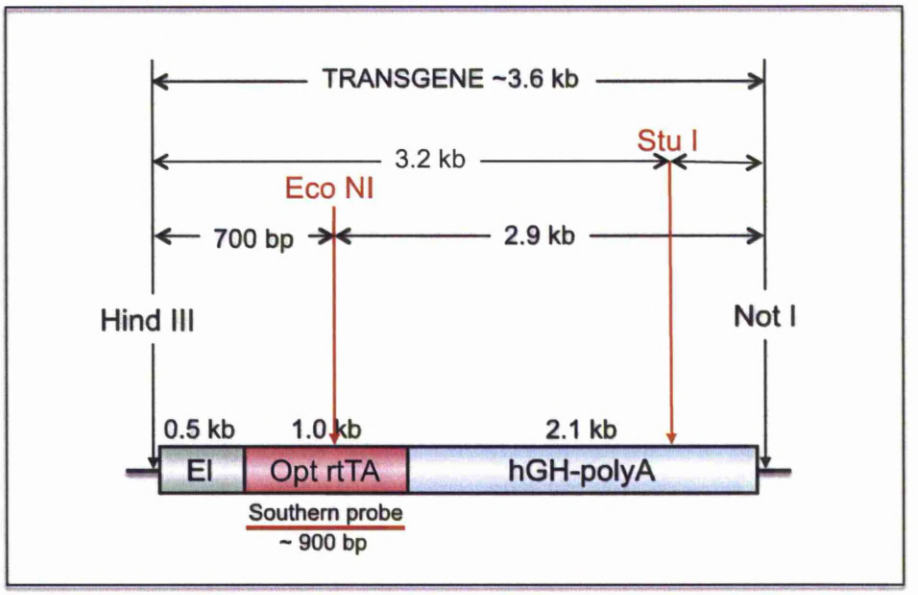

Fig 4.3 pBEG OptrtTA transgene

Schematic diagram representing the pBEG OptrtTA transgene. Shown are the *Hind*III and *Not*I **digestion sites used to liberate the transgene.** The *Stu*I site is cut when digesting genomic DNA and the Southern probe is generated by PCR. Also shown **is the position of the** restriction site *Eco*NI, **which was initially used for genomic digestion.** (El, elastase I promotor. hGH, human growth hormone).

4.2.4 Screening for pBI-G wtPRSS1HA

11 animals were generated from the first round of microinjections but Southern screening did not identify a founder animal.

21 animals were generated from the second round of microinjections and genomic DNA extracted from ~1cm tailclips of each animal. The DNA was digested using the *Stu*I restriction endonuclease and then samples were subjected to agarose gel electrophoresis and transferred to a nitrocellulose membrane (see section 2.13). The membrane was hybridised with a specific radionucleotide labelled probe to identify potential founder animals as described in section 4.2.2. A positive founder animal, number 20 (female), was identified (see figure 4.4). The Southern blot suggested that the transgene had integrated at multiple sites, given that there are more than two bands of a different size to the transgene. The intensity of the transgene band was similar to the 1 copy control and certainly less than the 10x copy control.

The founder animal was backcrossed onto wild type mice with the aim of generating a litter of mice carrying the transgene. The first generation litter of 19 animals was screened by the Southern blot technique and yielded three positive animals, 7.1, 8.3, and 9.2, showing a blot pattern similar to that of the founder animal (see figure 4.5).

Each of these animals were backcrossed onto wild type mice to sufficiently expand the transgenic strain and then mated with other positive animals to eventually generate both homozygous transgenic mice.

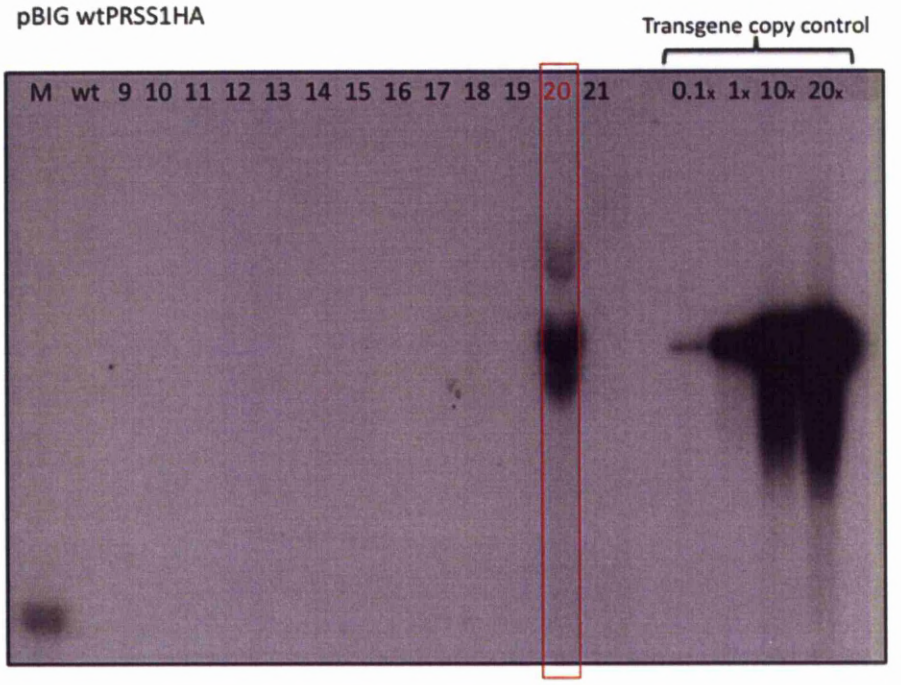

Fig 4.4 Southern Blot of pBI-G wtPRSS1HA animals

10μg genomic DNA (animals 9-21) digested using *Stu*I then run on a 0.7% w/v agarose gel and transferred to nitrocellulose membrane. Presence of transgene detected by 50μCi [α-^{32}P] radiolabelled probe demonstrated a positive animal 20. Transgene copy controls allow approximation of the number of transgenes integrated into murine genome. (M, marker; wt, wild type).

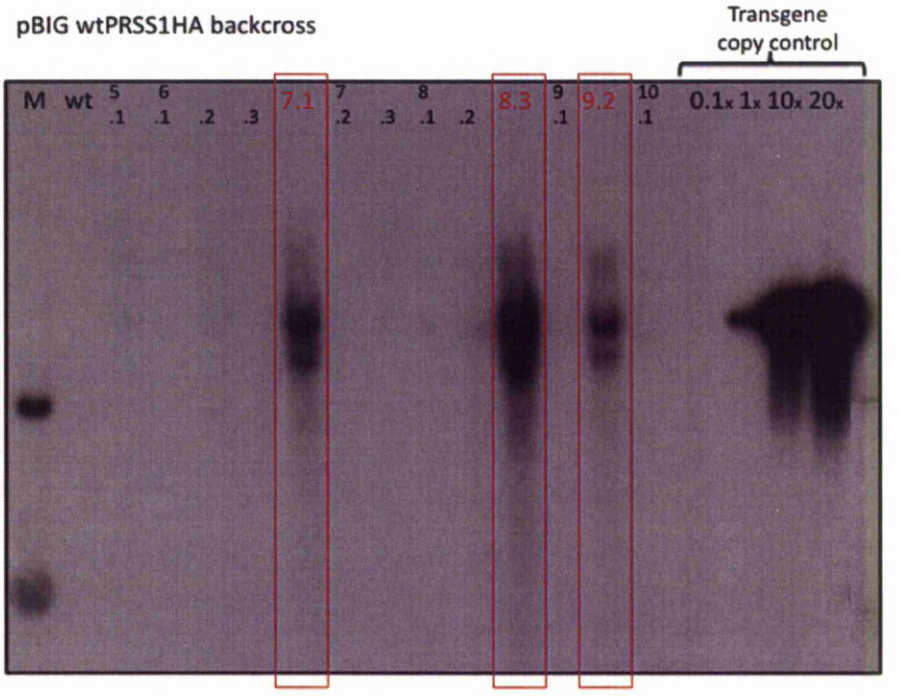

Fig 4.5 Southern Blot of pBI-G wtPRSS1HA backcross animals

10μg genomic DNA (animals 5.1-10.1) digested using *Stu*I then run on a 0.7% w/v agarose gel and transferred to nitrocellulose membrane. Presence of transgene detected by 50μCi [α-^{32}P] radiolabelled probe demonstrated positive animals 7.1, 8.3 & 9.2. Transgene copy controls allow approximation of the number of transgenes integrated into murine genome. (M, marker; wt, wild type).

4.2.5 Screening for pBI-G PRSS1HA R122H

18 animals were generated from the first round of microinjections and screened by my predecessor, Catherine Merriman. This litter identified two possible founders – animal 6 (female) and animal 14 (female)

These potential founder animals were backcrossed with wild type mice producing a total litter of 54 animals. These animals were extensively screened by me, but failed to show positive animals. The litter was re-clipped and screened but again no positive animals were identified. The original DNA from founder animal 6 was screened and failed to show the presence of the transgene.

5 animals were generated from a second round of microinjections and genomic DNA extracted from ~1cm tailclips of each animal. The DNA was digested using the *Stu*I restriction endonuclease and the samples were subjected to agarose gel electrophoresis then transferred to nitrocellulose membrane (see section 2.13). The membrane was hybridised with a specific radionucleotide labelled probe to identify potential founder animals as described in section 4.2.2. Two possible founder animals – animal 21 (male) and animal 22 (female) were identified (see fig 4.6). The Southern blot suggested that the transgene had integrated at multiple sites in animal 22 and the band intensity for both positive animals was less than the 10x copy control.

The founder animals were backcrossed onto wild type mice producing a total litter of 33 animals. These animals were analysed by Southern blot screening and resulted in identification of a further four positive animals, 16.1, 18.1, 18.4, and 23.2 (see figure 4.7A&B).

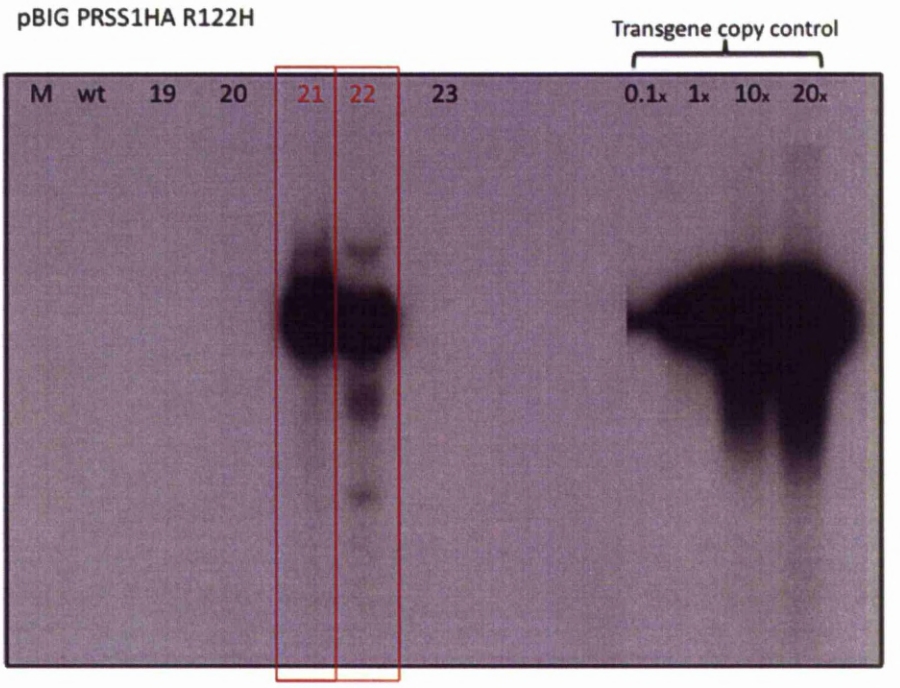

Fig 4.6 Southern Blot of pBI-G PRSS1HA R122H animals

10μg genomic DNA (animals 19-23) digested using *Stu*I then run on a 0.7% w/v agarose gel and transferred to nitrocellulose membrane. Presence of transgene detected by 50μCi [α-^{32}P] radiolabelled probe demonstrated positive animals 21 & 22. Transgene copy controls allow approximation of the number of transgenes integrated into murine genome. (M, marker; wt, wild type).

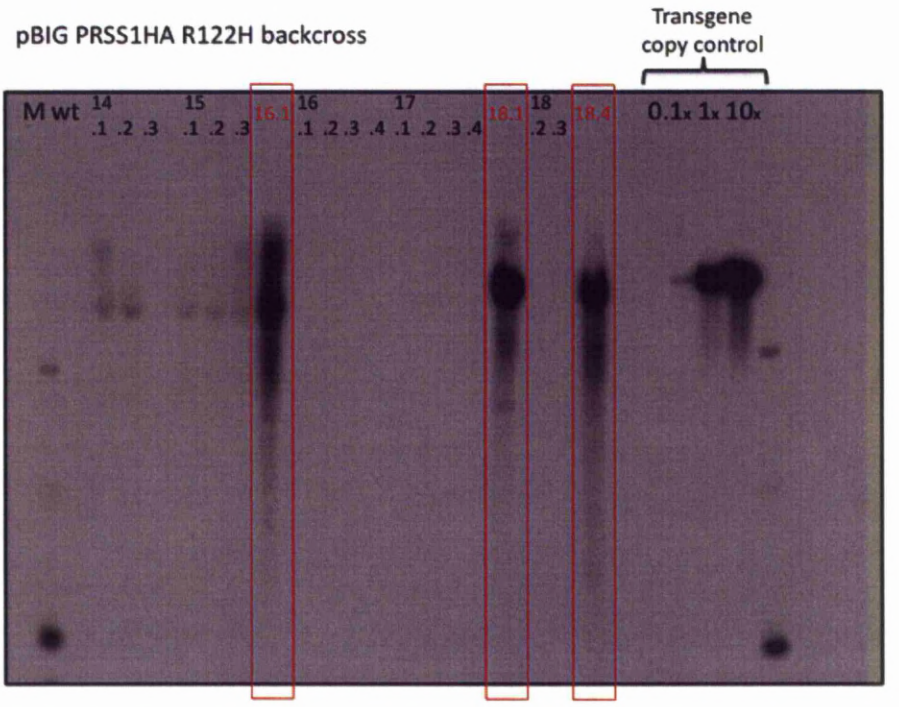

Fig 4.7A Southern Blot of pBI-G PRSS1HA R122H backcross animals

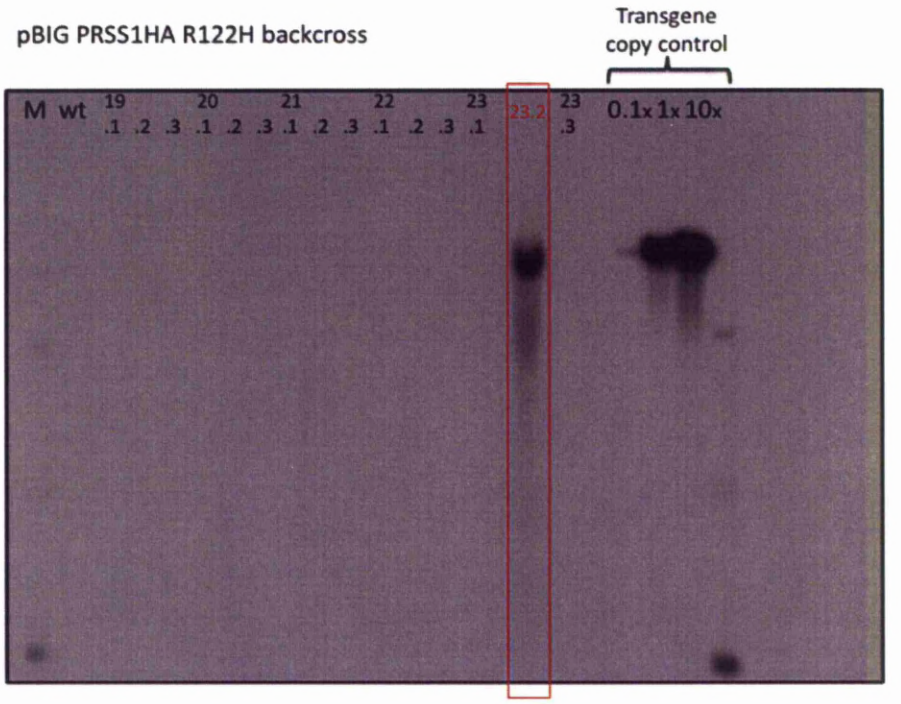

Fig 4.7B Southern Blot of pBI-G PRSS1HA R122H backcross animals

10μg genomic DNA (animals 14.1-23.3) digested using *Stu*I then run on a 0.7% w/v agarose gel and transferred to nitrocellulose membrane. Presence of transgene detected by 50μCi [α-^{32}P] radiolabelled probe demonstrated positive animals 16.1, 18.1, 18.4 (A) and 23.2 (B). Transgene copy controls allow approximation of the number of transgenes integrated into murine genome. (M, marker; wt, wild type).

4.2.6 Screening for pBI-G PRSS1HA N29I

9 animals were generated from the first round of microinjections and genomic DNA extracted from ~1cm tailclips of each animal. The DNA was digested using the *Stu*I restriction endonuclease and analysed by Southern blotting as described in section 4.2.5. Two possible founders animals –animal 2 and animal 9 were identified (see figure 4.8). The potential founder animals were backcrossed onto wild type mice. Subsequent screening of offspring from founder animal 2 failed to show positive animals and the founders were re-clipped and analysed. Unfortunately, animal 2 did not demonstrate the presence of the transgene.

Founder animal 9 was back-crossed and generated a litter of 14 animals. Screening of these animal by Southern blot identified three positive second generation animals; animal 9 (male), animal 11 (female), animal 12 (female) (see figure 4.9). The positive animals had a similar pattern on the Southern blot as the parent animal, however, due to degradation during storage of the transgene used as the copy control, the band intensity could not be reliably assessed.

Each of these animals was backcrossed onto wild type mice to sufficiently expand the transgenic strain and then mated with other positive animals to eventually generate homozygous transgenic mice.

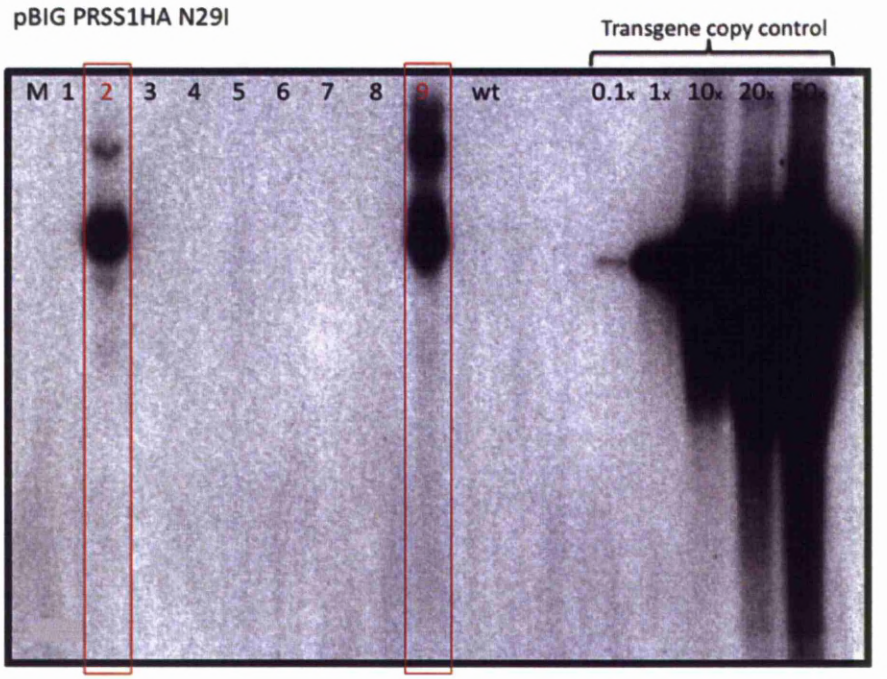

Fig 4.8 Southern Blot of pBI-G PRSS1HA N29I animals

10μg genomic DNA (animals 1-9) digested using *Stu*I then run on a 0.7% w/v agarose gel and transferred to nitrocellulose membrane. Presence of transgene detected by 50μCi [α-^{32}P] radiolabelled probe demonstrated positive animals 2 & 9. Transgene copy controls allow approximation of the number of transgenes integrated into murine genome. (M, marker; wt, wild type).

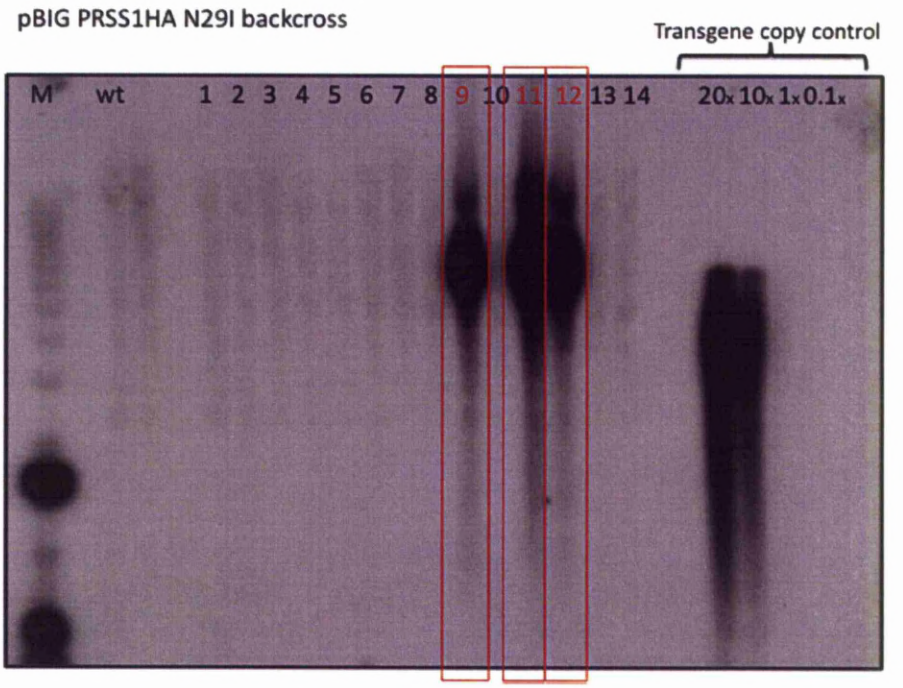

Fig 4.9 Southern Blot of pBI-G PRSS1HA N29I backcross animals

10μg genomic DNA (animals 1-14) digested using *Stu*I then run on a 0.7% w/v agarose gel and transferred to nitrocellulose membrane. Presence of transgene detected by 50μCi [α-^{32}P] radiolabelled probe demonstrated positive animals 9, 11 & 12. Transgene copy controls allow approximation of the number of transgenes integrated into murine genome. (M, marker; wt, wild type).

4.2.7 Screening for pBEG OprtTA

68 animals were generated over two rounds of microinjections and genomic DNA extracted from ~1cm tailclips of each animal. The DNA was digested using the *Eco*NI restriction endonuclease analysed by Southern blotting. Screening of these animals did not identify any positive founder animals.

Generation of the pBEG OptrtTA animal was imperative for the inducible model, as without this animal we would be unable to generate the required compound animal that would be able to transcribe our genes of interest in an inducible manner. Given the success of generating the pBI-G PRSS1HA cohort of animals, and the failure of repeated attempts of identifying a positive pBEG OptrtTA animal from a reasonable large potential group, the pBEG OptrtTA plasmid was cloned again from scratch and commercially sequenced (see section 3.2.5). This revealed that there were two *Eco*NI restriction sites present in the transgene as opposed to the one site that the screening strategy had been based upon (see figure 4.3). The strategy was therefore altered with the restriction endonuclease *Eco*NI being replaced by *Stu*I.

12 animals were generated from the third round of microinjections. Screening of this litter demonstrated a positive female founder animal number 193.1 (see figure 4.10). The band intensity suggested the transgene

had integrated more than once and less than ten times into the murine genome.

The founder animal was backcrossed with a wild type animal in order assess integration of the transgene into the germ line and to expand the lineage of the transgene.

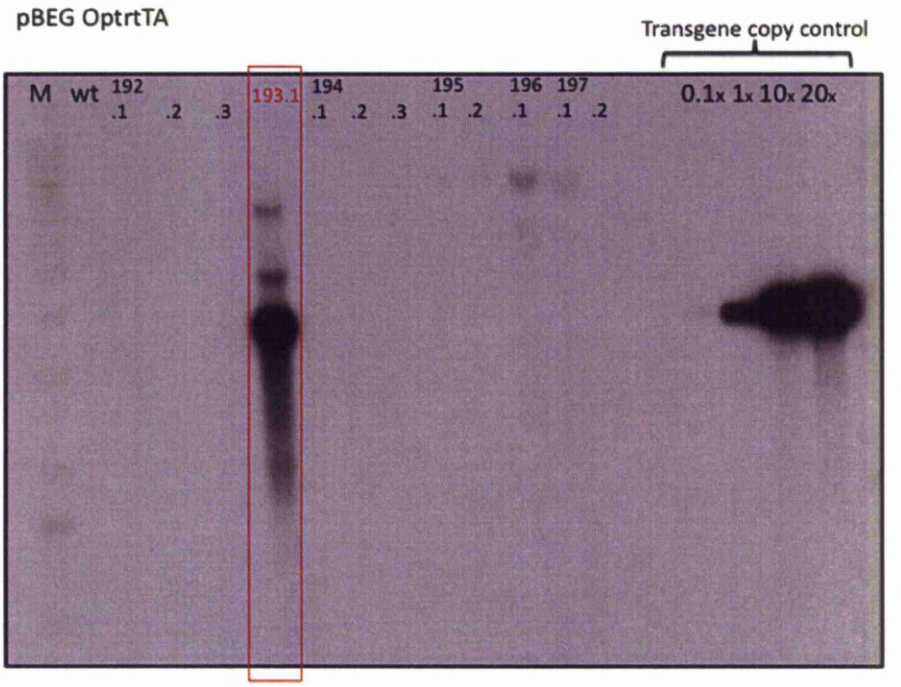

Fig 4.10 Southern Blot of pBEG OptrtTA animals

10μg genomic DNA (animals 192.1-197.2) digested using *Stu*I then run on a 0.7% w/v agarose gel and transferred to nitrocellulose membrane. Presence of transgene detected by 50μCi [α-^{32}P] radiolabelled probe demonstrated a positive animal 193.1. Transgene copy controls allow approximation of the number of transgenes integrated into murine genome. (M, marker; wt, wild type).

4.3 Summary

The aim of the experiments described above was to generate genetically modified mice with 'inducible' protein expression, based on the Tet-On system. The first construct, based on the pBI-G bidirectional vector, would carry the gene of interest i.e. wt PRSS1 or one of the two mutants and the second construct, which expresses the inducer protein OptrtTA, would be driven by an upstream rat elastase 1 promoter. Cross breeding of these two strains of mice would provide a compound mouse strain that could, upon feeding with doxycycline, be induced to transcribe the gene of interest in the acinar cells of the pancreas.

Analysis of the offspring microinjected with the pBI-G wtPRSS1HA transgene identified a positive animal number 20. This animal was then backcrossed and gave rise to three positive offspring (animals 7.1, 8.3, & 9.2). Two founder animals were successfully identified carrying the pBI-G PRSS1HA R122H transgene (animals 21 & 22) and the pBI-G PRSS1HA N29I transgene (animals 2 & 9). These strains were also backcrossed with wild type non-transgenic animals to generate positive offspring (animals 16.1, 18.1, 18.4, 23.2 and animals 9, 11, 12 respectively).

	Founder animals	Second generation
pBI-G wtPRSS1HA	20	7.1, 8.3, 9.2
pBI-G PRSS1HA R122H	21, 22	16.1, 18.1, 18.4, 23.2
pBI-G PRSS1HA N29I	2, 9	9, 11, 12
pBEG OptrtTA	193.1	

Fig 4.11 Table of positive founder animals

A table showing the positively identified founder and second generation of transgenic animals for the strains pBI-G wtPRSS1HA, pBI-G PRSS1HA R122H, pBI-G PRSS1HA N29I and pBEG OptrtTA.

The animals positively identified carrying the transgenes are summarised in figure 4.11.

Generation and identification of mice with integration of the essential pBEG OptrtTA transgene into their genome was far less successful. Despite the creation of a large cohort of potentially positive animals, screening failed to identify integration of the transgene into the murine genome. This failure was of great concern, as the pBEG OptrtTA transgene would be crucial in driving expression of the PRSS1 transgenes in the final compound mouse. Due to these difficulties, it was decided that the plasmid should be cloned again and then re-sequenced prior to microinjection. The sequencing revealed that there were two *Eco*NI site located within the transgene and digesting the murine genome with this restriction endonuclease would result in a fragment that would not run at the expected position, and may in fact be too small to detect, on a Southern blot. New animals were created by microinjection of the new pBEG OprtTA transgene and the restriction endonuclease *Stu*I was used to digest the murine genome. This led to the identification of a positive founder animal number 193.1.

All positively identified animals were extensively backcrossed to generate a large pool of transgenic animals for each of the individual strains. Eventually, compound mice would be generated carrying the pBEG OptrtTA transgene and one of the PRSS1 based transgenes. These animals

would then be analysed to determine whether they develop a phenotype, either spontaneously or after a pharmacological challenge, characteristic of pancreatitis.

Chapter 5

5 GENERATION OF MICE WITH CONSTITUTIVELY EXPRESSED TRANSGENES

5.1 Introduction

As a consequence of the difficulties encountered in establishing a genetically modified mouse carrying the pBEG OptrTA transgene, a different strategy of generating genetically modified mice stably expressing human cationic trypsinogen was developed. As opposed to the inducible model, which required a compound mouse with the PRSS1 and OptrtTA transgenes integrated into the murine genome, a 'non-inducible' model was designed. In this model the human cationic trypsinogen gene or one of the two mutants (R122H or N29I) would be directly driven by the rat elastase I promoter. This model would therefore allow constitutive expression of the transgene, specifically in the acinar cells of the mouse pancreas.

5.2 Results

5.2.1 Preparation of transgenes

The transgenes for pBEG wtPRSS1HA, pBEG PRSS1HA R122H and pBEG PRSS1HA N29I were liberated in a similar fashion, by digesting the appropriate constructs with the restriction endonucleases *Hind*III and *Not*I. The samples were subjected to agarose gel electrophoresis and the 3.4kb

band containing the transgene was extracted (as described in section 2.10.6) (see figure 5.1).

5.2.2 Preparation of Probe for Southern Blotting

In order to identify successful integration of the transgene into the murine genome, the litter from microinjected animals was screened using a Southern blot technique (see section 2.13). This required a radionucleotide labelled probe to allow specific hybridisation with a corresponding sequence on the integrated transgene.

The screening of animals microinjected with the transgenes pBEG wtPRSS1HA, pBEG PRSS1HA R122H or pBEG PRSS1HA N29I was performed using the same probe. The probe was prepared by digesting the pBEG wtPRSS1HA plasmid with *Bam*HI and the sample was run on a 0.7% w/v GTG agarose gel. The 800bp band, representing the probe, was extracted and prepared as described in section 2.13.

Alternatively, the probe could be generated by PCR, using the 5' PRSS1 and 3' PRSS1 primers and pBEG wtPRSS1HA plasmid as the template DNA (see figure 5.1).

5.2.3 Establishing Founder Animals

After microinjection of the mouse embryos, the newborn pups were screened by Southern blot analysis to identify founder animals. The mice that were found to be positive carriers of the transgene were then

backcrossed with wild type, non-transgenic mice. This allowed each lineage to be expanded, and also, enabled the segregation of the transgene if it had integrated into multiple sites of the murine genome. Wild type murine DNA was always digested and loaded with the samples to exclude false positive identification. Transgene DNA was loaded at known concentrations to help estimate the number of copies integrated (see section 2.13).

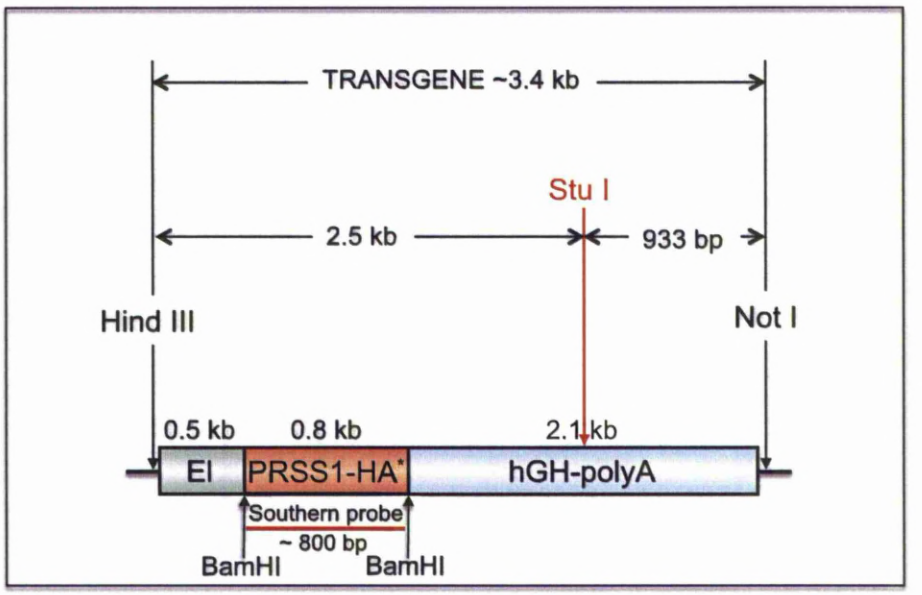

Fig 5.1 pBEG wtPRSS1HA transgene

Schematic diagram representing the pBEG OptrtTA transgene (*may also represent PRSS1HA R122H or PRSS1HA N29I). Shown are the *Hind*III and *Not*I digestion sites used to liberate the transgene. The *Stu*I site is cut when digesting genomic DNA and the Southern probe is liberated by digestion with *Bam*HI or PCR. (EI, elastase I promotor. hGH, human growth hormone).

5.2.4 Screening for pBEG wtPRSS1HA

14 animals were generated from the first round of microinjections and genomic DNA extracted from ~1cm tailclips of each animal. The DNA was digested using the *Stu*I restriction endonuclease and then samples were subjected to agarose gel electrophoresis and transferred to nitrocellulose membrane (see section 2.12). The membrane was hybridised with a specific radionucleotide labelled probe to identify potential founder animals as described in section 5.2.2. A positive founder animal, number 13 (female), was identified (see figure 5.2). The Southern blot suggested that the transgene had integrated at least at one site, whilst the intensity of the transgene band was greater than 1 copy control and less than the 10x copy control.

The founder animal was backcrossed onto wild type mice with the aim of generating a litter of mice carrying the transgene. Each of these positive animals was backcrossed onto wild type mice to sufficiently expand the transgenic strain and then mated with other positive (heterozygous) animals to eventually generate homozygous transgenic mice.

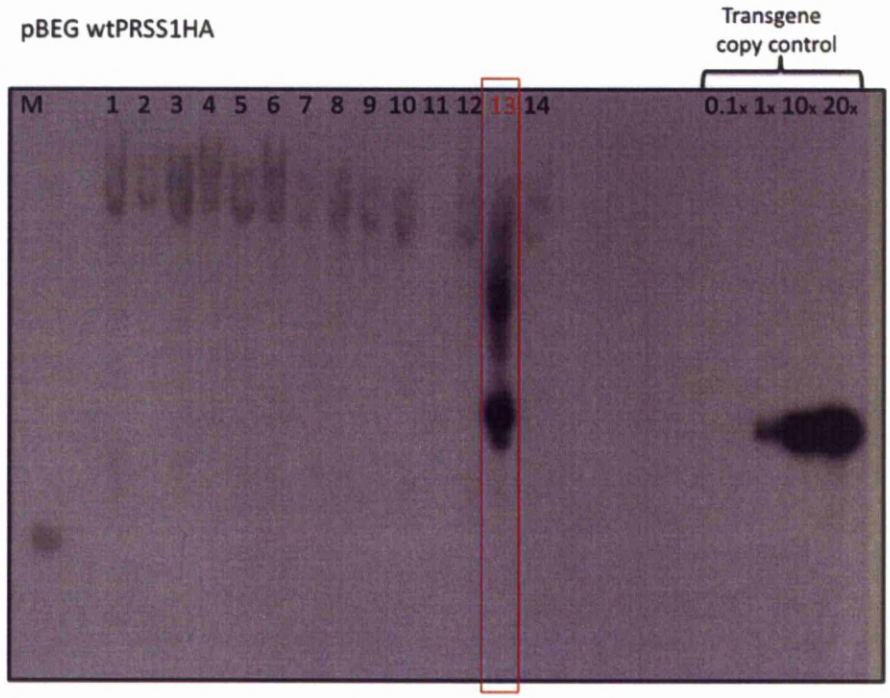

Fig 5.2 Southern Blot of pBEG wtPRSS1HA animals

10μg genomic DNA (animals 1-14) digested using *Stu*I then run on a 0.7% w/v agarose gel and transferred to nitrocellulose membrane. Presence of transgene detected by 50μCi [α-^{32}P] radiolabelled probe demonstrated a positive animal 13. Transgene copy controls allow approximation of the number of transgenes integrated into murine genome. (M, marker; wt, wild type).

5.2.5 Screening for pBEG PRSS1HA R122H

8 animals were generated from the first round of microinjections but screening this litter failed to identify any positive animals

26 animals (numbers 38.1-46.3) were generated from a second round of microinjections and genomic DNA extracted and screened as described in section 5.2.4. A possible founder animal 39.1 (female) was identified (see fig 5.3A). The Southern blot suggested that the transgene had integrated at least at one site and the band intensity was similar to the 10x copy control.

The founder animal was backcrossed onto wild type mice with the aim of generating a litter of mice carrying the transgene. Each of these positive animals was backcrossed onto wild type mice to sufficiently expand the transgenic strain and then mated with other positive (heterozygous) animals to eventually generate homozygous transgenic mice.

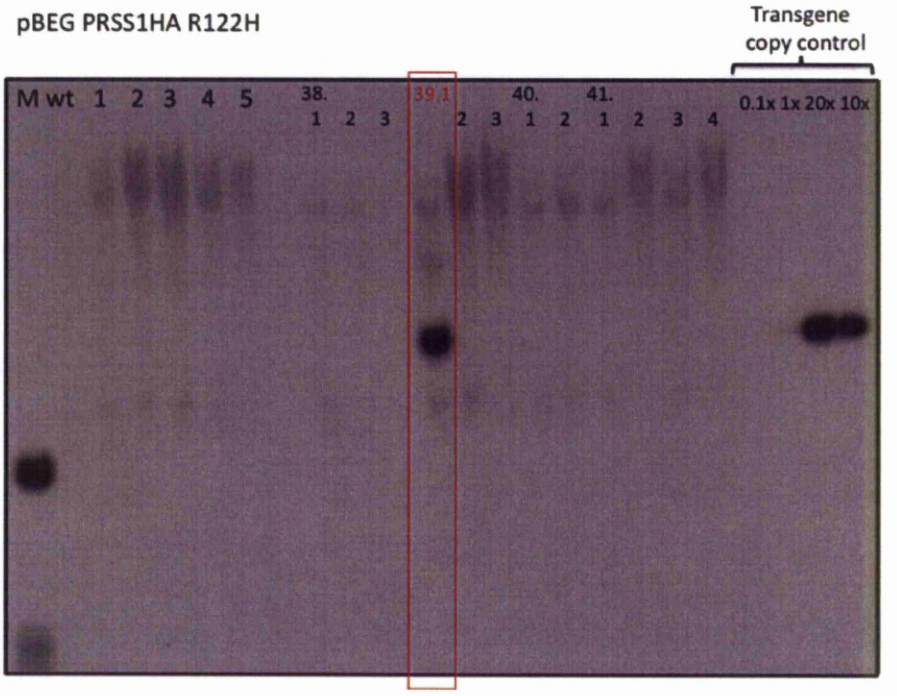

Fig 5.3A Southern Blot of pBEG PRSS1HA R122H animals

Fig 5.3B Southern Blot of pBEG PRSS1HA R122H animals

10μg genomic DNA (animals 1-5; 38.1-41.4 (A) & (B) animals 42.1 – 46.3) digested using *Stu*I then run on a 0.7% w/v agarose gel and transferred to nitrocellulose membrane. Presence of transgene detected by 50μCi [α-^{32}P] radiolabelled probe demonstrated a positive animal 39.1 (A). Transgene copy controls allow approximation of the number of transgenes integrated into murine genome. (M, marker; wt, wild type).

5.2.6 Screening for pBEG PRSS1HA N29I

19 animals were generated from the first round of microinjections and genomic DNA extracted and screened as described in section 5.2.4.

Three possible founder animals 10, 14, 19 were identified (see fig 5.4). The Southern blot suggested that the transgenes had integrated at least at one site. The band intensity of founder animal 19 was similar to the 1x copy control, the band intensity of founder animal 14 was greater than the 1x but less than the 10x copy control, whilst the band intensity of founder animal 10 was greater than 10x and similar to the 20x copy control.

Again, the founder animals were backcrossed onto wild type mice with the aim of generating a litter of mice carrying the transgene. Each of these positive animals was backcrossed onto wild type mice to sufficiently expand the transgenic strain and then mated with other positive (heterozygous) animals to eventually generate homozygous transgenic mice.

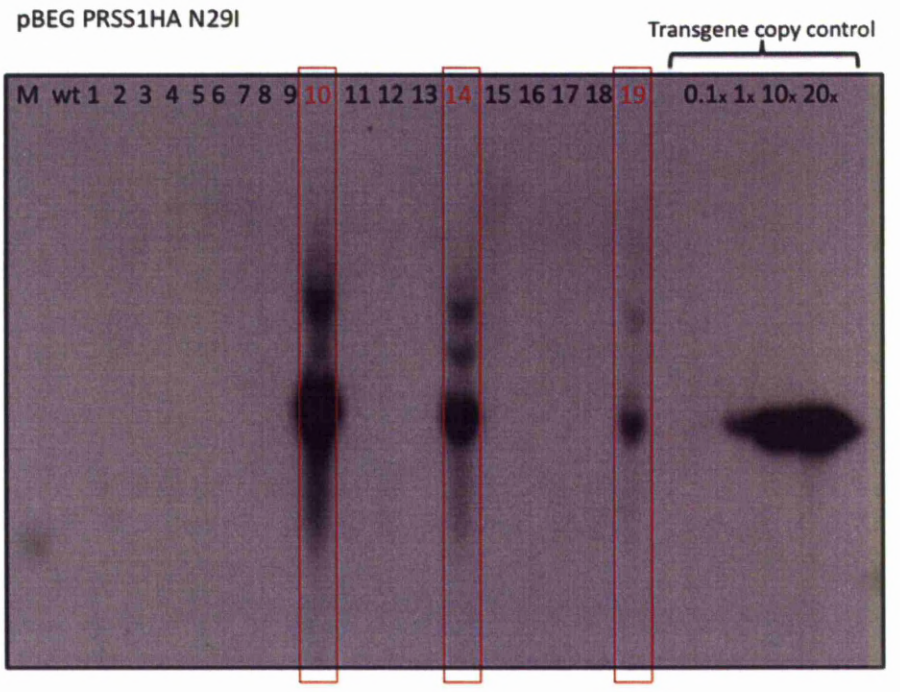

Fig 5.4 Southern Blot of pBEG PRSS1HA N29I animals

10μg genomic DNA (animals 1-19) digested using *Stu*I then run on a 0.7% w/v agarose gel and transferred to nitrocellulose membrane. Presence of transgene detected by 50μCi [α-^{32}P] radiolabelled probe demonstrated positive animals 10, 14 & 19. Transgene copy controls allow approximation of the number of transgenes integrated into murine genome. (M, marker; wt, wild type).

5.3 Summary

As a consequence of the difficulties encountered in establishing a genetically modified mouse carrying the pBEG OptrTA transgene, a critical component of the inducible model, a different strategy of generating genetically modified mice stably expressing human cationic trypsinogen was developed. The aim was to generate genetically modified mice in which the rat elastase driven genes of interest were integrated into the murine genome. This model would allow constitutive expression of the PRSS1 based transgenes within the acinar cells of the mouse pancreas.

Analysis of the offspring from the first round of microinjections with the pBEG wtPRSS1HA transgene identified a founder, animal 13, from a litter of fourteen animals. Two round of microinjection were completed, generating a total litter of 34 animals, prior to the identification of a positive founder, animal 39.1, for the pBEG PRSS1HA R122H transgene. Microinjection of the pBEG PRSS1HA N29I transgene proved to be most successful as the first round generated a litter of 19 mice of which three were potential founder animal; animals 10, 14, & 19. These animals are summarised in figure 5.5.

	Founder animals
pBEG wtPRSS1HA	13
pBEG PRSS1HA R122H	39.1
pBEG PRSS1HA N29I	10, 14, 19

Fig 5.5 Table of positive founder animals

A table showing the positively identified founder and second generation of transgenic animals for the strains pBEG wtPRSS1HA, pBEG PRSS1HA R122H, pBEG PRSS1HA N29I.

The positive animals from each cohort were paired with wild type non-transgenic mice to expand the pool of genetically modified animals. The positive animals would all be dated at birth and eventually analysed at different time points to determine whether they develop features of pancreatitis.

The analysis of the animals for each of the transgenic strain began once adequate numbers had been generated in each cohort. The characterisation of the phenotype developed in each transgenic strain and the consequences of challenging these animals with widely recognised techniques to induce acute pancreatitis, were performed after completion of the laboratory work undertaken for this MD project, and is therefore outside the scope of this thesis.

Chapter 6

6 ASSAY TO ASSESS TRYPSIN ACTIVITY

6.1 Introduction

Having created expression plasmids with the aim of expressing human cationic trypsinogen, or one of the two common mutant alleles, it was imperative to ensure that the proteins were expressed and that they retained their enzymatic activity. As described in chapter 3, the *PRSS1* gene had been cloned into a pCEP plasmid with expression driven by a CMV promoter. This would allow transient transfection of cultured cell lines to determine whether protein expression was detectable. If protein expression was confirmed, then cell lysates could be subjected to an *in vitro* assay to assess trypsin activity. The assay would be based on the ability of trypsin to cleave a fluorogenic substrate (Rhodamine-110), resulting in peak excitation and emission wavelengths of 498nm and 521nm respectively, and the fluorescence measured by a spectrophotometer.

6.2 Results

6.2.1 Detection of human cationic trypsinogen

The genes designed to be expressed in the transgenic mice were all labelled with a HA tag in an effort to allow identification as we were unsure whether a specific antibody directed against cationic trypsinogen would be sufficiently sensitive or specific at detection of the transgenic protein in a

backdrop of native murine trypsinogens. However, cDNA were developed with and without a HA tag and cloned into the pCEP vector to assess expression.

Prior to this, we tested a polyclonal and four monoclonal antibodies raised against human cationic trypsinogen. As positive controls, serial dilutions of recombinant human trypsin, ranging from 1000ng to 3ng, were run on a SDS-PAGE gel and transferred to a nitrocellulose membrane then probed with each of the antibodies (as described in section 2.8). The polyclonal antibody and two of the monoclonal antibodies failed to detect any protein at the tested concentrations. The monoclonal antibody anti-PRSS1 13404 was shown to be the most sensitive, detecting a visible band corresponding to as little as 15ng of recombinant human trypsin (see figure 6.1).

H1299 cells were transiently transfected (as described in section 2.7) with the pCEP-based plasmids to establish expression of the proteins of interest. WB analysis (as described in section 2.8) using the anti-HA antibody and the anti-PRSS1 (13404) antibody would allow comparison of the relative sensitivity of the individual antibodies, whilst simultaneously confirming expression *in vitro*.

As shown in figure 6.2, expression of the proteins is demonstrated using both the anti-PRSS1 and also the anti-HA antibody. Mock transfection using the empty pCEP vector acted as a negative control (seen in the first

lane figure 6.2). The untagged proteins run lower than their tagged counterparts as seen using anti-PRSS1, consistent with the slight difference in molecular weight. The anti-HA antibody appears to be slightly more sensitive relative to anti-PRSS1. Loading of each lane is similar as shown by the actin bands, whilst the β-gal levels reveal variable transfection efficiency.

This data suggests that H1299 can be transiently transfected with the pCEP-based plasmids and that the proteins of interest are expressed at detectable levels. Detection of as little as 15ng of protein appears to be possible using the anti-PRSS1 antibody whilst the anti-HA antibody is slightly more sensitive.

6.2.2 Optimisation of the trypsin assay

An assay to assess the enzymatic activity of the tagged forms of human cation trypsinogen was developed once detectable expression had been shown, in transiently transfected cells in culture, by western blot analysis.

Prior to applying the assay to transfected cell lysates, the principles were assessed using a commercially available high-grade recombinant human trypsin. 20ng/ml of recombinant trypsin was spiked into 0.1M Tris-HCl (pH 8.0), with 5μM BZiPAR (a Rhodamine-110 based substrate allowing fluorescence after cleavage by trypsin) with or without the addition of 1mM $CaCl_2$ and 2ng/ml of enterokinase (1ml final volume). The assay was

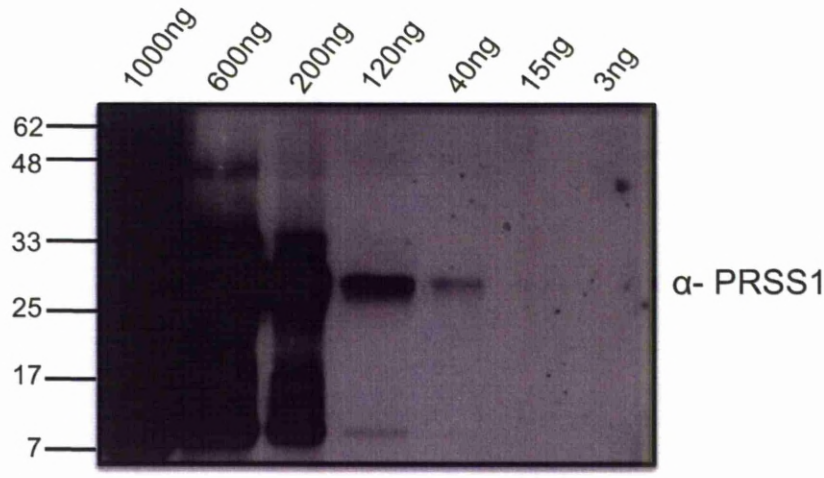

Fig 6.1 Detection of recombinant human cationic trypsinogen

Serial dilutions of recombinant human trypsin ranging from 1000ng to 3ng were run on 12% SDS polyacrylamide gel and transferred to nitrocellulose membrane and probed with anti-PRSS1 (13404) antibody. Detection of as little as 15ng of protein was achieved.

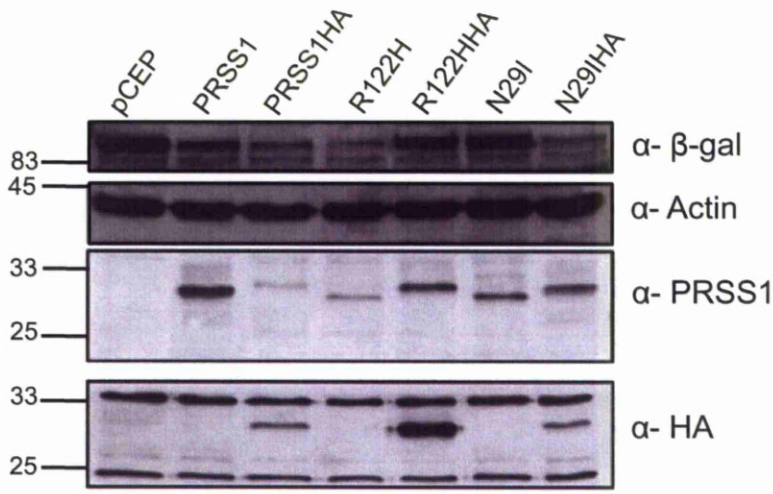

Fig 6.2 Western blots of human cationic trypsinogen

Transiently transfected H1299 cells with indicated pCEP-based plasmids (as shown per lane) were subjected to western bloting according to Laemelli (1976), and probed with the relavent antibodies.

performed (as described in section 2.9) over a time course of 60 minutes, at room temperature, with measurements taken every 10 minutes.

Enterokinase by itself was used as a negative control and generated the weakest activity with regards to cleavage of the Rhodamine-110 substrate (see figure 6.3). Recombinant trypsin activity was augmented by the addition or either 1mM calcium chloride or 2ng/ml enterokinase. The activity of recombinant trypsin was highest with the addition of 2ng/ml enterokinase and 1mM calcium chloride (see figure 6.3), suggesting that there may be some trypsinogen present within the mixture that required enterokinase mediated activation. The assay demonstrated that 20ng of recombinant trypsin could cleave the fluorogenic substrate at a level detectable by the spectrophotometer over the course of an hour.

H1299 cells were transiently transfected with pCEP wtPRSS1HA and either mock transfected with the empty pCEP vector or left untransfected in order to provide negative controls (see section 2.7). Cells were harvested, lysed using SLIP buffer without protease inhibitors, and the total protein concentration calculated the Bradford method (see section 2.7). 50μg/ml of protein was loaded onto 12% SDS polyacrlamide gels for western blotting (see section 2.8) and probed with anti-HA antibody. 150μg/ml total protein was spiked into 0.1M Tris-HCl (pH 8.0) for the trypsin assay (see section 2.9). 2ng/ml enterokinase, 1mM $CaCl_2$ and 5μM

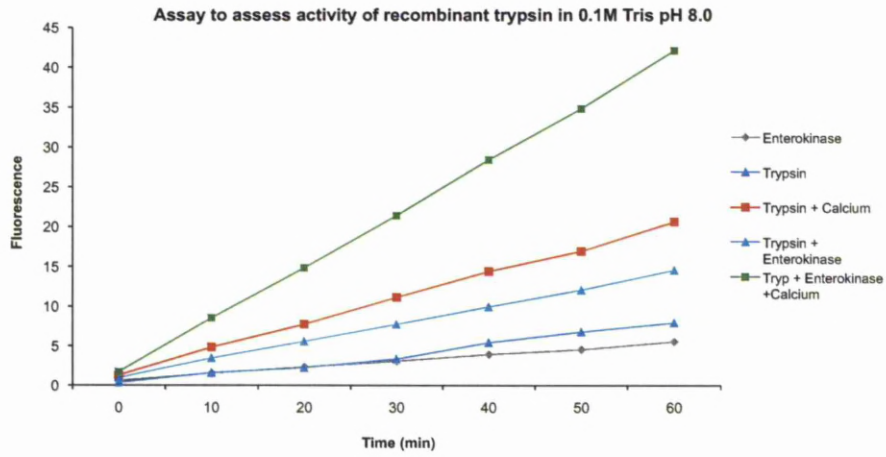

Fig 6.3 Assay to assess activity of recombinant human trypsin

20ng/ml of recombinant human trypsin was spiked into 0.1M Tris-HCl (pH 8.0) with or without the addition of 2ng/ml enterokinase and 1mM $CaCl_2$. 5μM (final concentration) of the Rhodamine-110 based substrate BZiPAR was added to each reaction and the reaction kinetics followed over the course of 60 minutes. Enterokinase (2ng/ml) was used as a control. The activity of recombinant trypsin was evident, especially in the presence of 2ng/ml enterokinase and 1mM $CaCl_2$. (Fluorescence measured in arbitary units).

BZiPAR were added to give a final volume of 1ml. The reaction kinetics were followed over a period of 60 minutes.

The experiment showed little to no difference in fluorescence between the negative controls and the pCEP wtPRSS1HA lysates (see figure 6.4). Western blotting confirmed detectable levels of wtPRSS1HA. The experiment was repeated but with similar results.

This raised the possibility that the tagged wtPRSS1HA protein was either not enzymatically functional or that there was inhibition of its activity in H1299 cells. The H1299 cells may have a native trypsin inhibitor or modify the protein to ameliorate its activity. To test this hypothesis, 20ng/ml recombinant trypsin was spiked into untransfected H1299 cell lysate and AR4-2J (derived from rat pancreas tumour cells) cell lysate. A positive control was set up by spiking recombinant trypsin into SLIP buffer to ensure this did not alter the enzymatic activity. As before, 2ng/ml enterokinase, 1mM $CaCl_2$ and 5μM BZiPAR were added to a final volume of 1ml in 0.1M Tris-HCl (pH 8.0) and the reaction kinetics were assessed for 60 minutes (see section 2.9).

Recombinant trypsin in SLIP buffer showed a dramatic ability to cleave the substrate BZiPAR, whilst its activity in H1299 cells was almost completely ameliorated (see figure 6.5). The activity of recombinant trypsin spiked

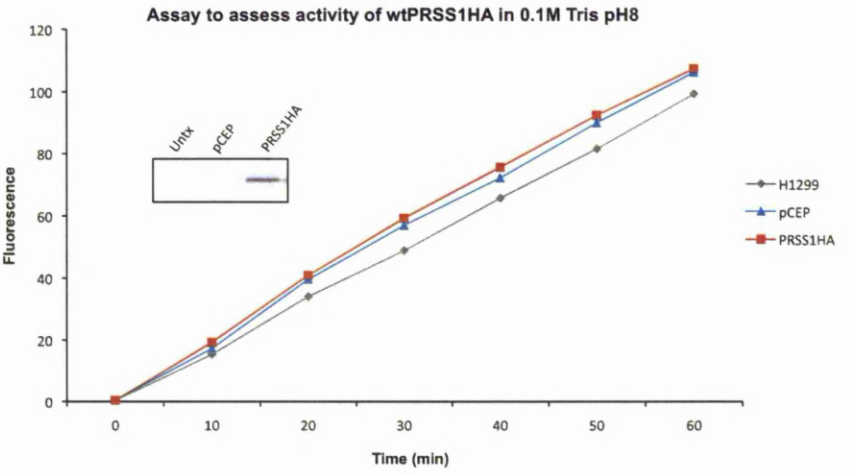

Fig 6.4 Assay to assess activity of wtPRSS1HA in cellular lysate

Transiently transfected H1299 cells were lysed in SLIP buffer without the addition of protease inhibitors. 150μg/ml of total protein was added to 0.1M Tris-HCl (pH 8.0) with 2ng/ml enterokinase and 1mM $CaCl_2$. 5μM (final concentration) of the Rhodamine-110 based substrate BZiPAR was added to each reaction and the reaction kinetics followed over the course of 60 minutes at 37°C. 50μg/ml of total protein was run on a 12% SDS polyacrylamide gel, transferred to nitrocellulose membrane and probed with anti-HA antibody (insert). No difference in activity was seen. (Fluorescence measured in arbitary units).

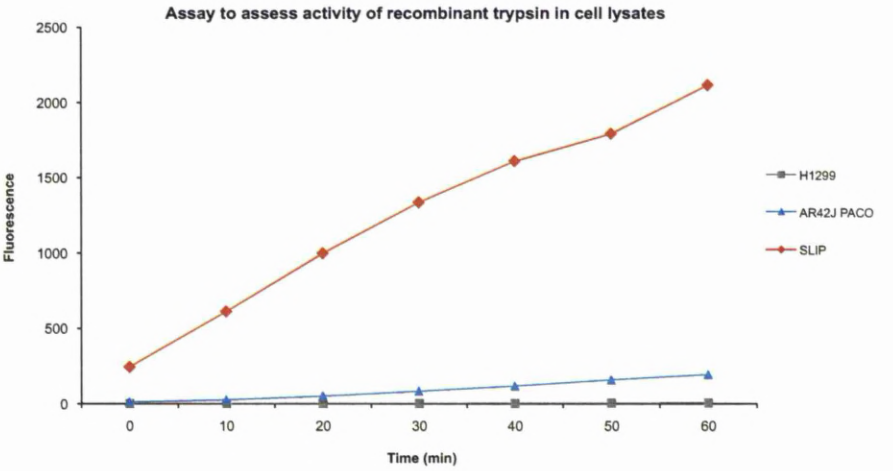

Fig 6.5 Assay to assess activity of recombinant trypsin in cell lysates

20ng/ml of recombinant human trypsin was spiked into either untransfected H1299 or AR4-2J cell lysates with 2ng/ml enterokinase and 1mM $CaCl_2$ in 0.1M Tris-HCl (pH 8.0). 5μM (final concentration) of the Rhodamine-110 based substrate BZiPAR was added to each reaction and the reaction kinetics followed over the course of 60 minutes at 37°C. Recombinant trypsin in SLIP buffer was a positive control (red line). The activity of recombinant trypsin was evident in SLIP buffer but inhibited by both cell lysates. (Fluorescence measured in arbitary units).

into the AR4-2J cell lysate, though severely inhibited, appeared to be less affected than in the H1299 cell lysate.

Having identified an inhibitory effect in cell lysates, which could render the trypsin assay obsolete, an extensive search was undertaken to find a cell line that did not exhibit this property or was least effective. We utilised cell lines in regular use in our laboratory derived from colon cancer, pancreatic cancer or renal cancers. Cell lines A498, ACHN, HEK 293T, 111, 115, 117 and 121 were derived from renal cell carcinomas, SW 480, SW 620, HCT+, HT29 and LOVO were derived from colorectal adenocarcinomas and MiaPaCa cell line from pancreatic cancer.

Again, 20ng/ml of recombinant trypsin was spiked into each different cell lysate with the other conditions as described above and in section 2.9. The activity of the recombinant trypsin was reduced in all cell lysates in relation to the activity achieved in SLIP buffer (see figure 6.6). The least inhibitory effect appeared to occur in the cell line ACHN and A498 both derived from renal cell cancer. In these cell lines the fluorescence at 60 minutes was just under 50% of the maximum achieved in SLIP buffer, suggesting that they may be the best candidates for further assay experiments.

However, at this time Sahin-Tóth et al published a paper describing a cell line, human embryonic kidney 293T (HEK 293T), amenable to transient

transfection with cationic trypsinogen, which then secreted the protein into the culture medium (Sahin-Toth, Kukor et al. 2006). This provided an opportunity to develop the trypsin assay to assess enzymatic activity of the proteins harvested from the culture medium (see section 2.9). In this way we would hope to eliminate the inhibitory effects that were encountered whilst using cell lysates.

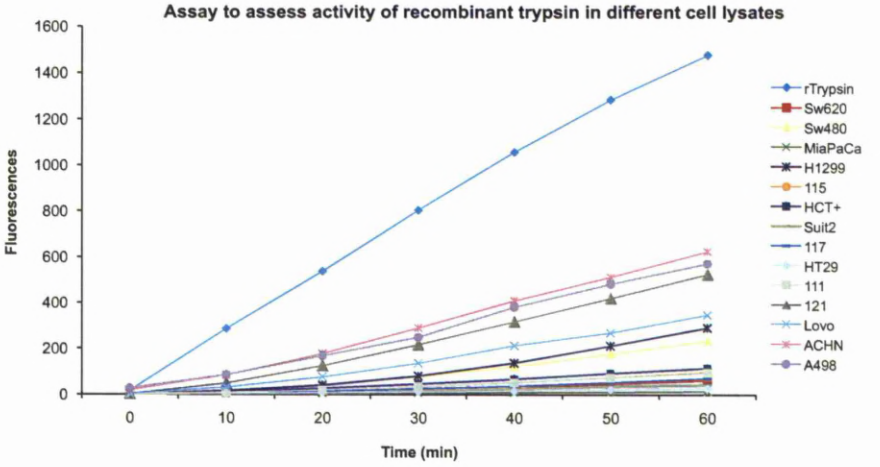

Fig 6.6 Activity of recombinant trypsin in various cell lysates

20ng/ml of recombinant human trypsin was spiked into different cell lysates as shown with 2ng/ml enterokinase and 1mM $CaCl_2$ in 0.1M Tris-HCl (pH 8.0). 5μM (final concentration) of the Rhodamine-110 based substrate BZiPAR was added to each reaction and the reaction kinetics followed over the course of 60 minutes at 37°C. Recombinant trypsin in SLIP buffer was a positive control (sky blue line; rTypsin). The activity of recombinant trypsin was evident in SLIP buffer but inhibited to varying degrees in the different cell lysates. Colorectal cell lines: SW 480, SW620, HCT+, HT29, LOVO. Renel cell lines: A498, ACHN, 111, 115,117, 121. Pancreas cell line MiaPaCa. (Fluorescence measured in arbitary units).

The HEK 293T cells were transiently transfected with pCEP wtPRSS1HA, or one of the mutant alleles, and the culture medium changed for a reduced serum medium Opti-MEM. Secretion of the proteins into the Opti-MEM medium would be subjected to assay. Prior to this it was necessary to determine whether the assay could be performed with recombinant human trypsin in Opti-MEM (see section 2.9.2).

20ng/ml recombinant trypsin with 2ng/ml enterokinase, 1mM $CaCl_2$ and 5μM BZiPAR were made to a final volume of 1ml in Opti-MEM (see section 2.9). The reaction kinetics were followed at 37°C for 60 minutes (see section 2.9.2). Negative controls were Opti-MEM without additives and Opti-MEM with 2ng/ml enterokinase and 1mM $CaCl_2$. Minimal activity, suggestive of substrate cleavage, was seen with either control (see figure 6.7) but was clearly seen with recombinant trypsin. This confirmed the suitability of Opti-MEM for use in the assay.

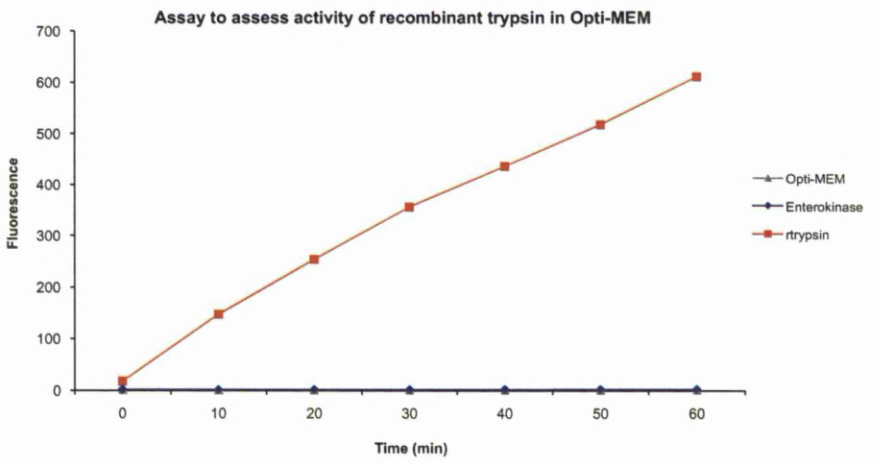

Fig 6.7 Assay to assess activity of recombinant trypsin in Opti-MEM

20ng/ml of recombinant human trypsin was spiked into Opti-MEM, a reduced serum culture medium, with 2ng/ml enterokinase 1mM $CaCl_2$, and 5μM BZiPAR (final concentrations) and the reaction kinetics followed over the course of 60 minutes at 37°C. Opti-MEM without additives (grey line) and Opti-MEM with 2ng/ml enterokinase and 1mM $CaCl_2$ (blue line) were negative controls. The activity of recombinant trypsin in Opti-MEM (red line) was evident whilst almost no activity was seen in either control. (Fluorescence measured in arbitary units).

6.2.3 Results of trypsin assay

HEK 293T cells were transiently transfected with pCEP wtPRSS1HA, mock transfected with empty pCEP vector or left untransfected. 12 hours after transfection the culture medium was replaced by Opti-MEM. A further 12 hours later the Opti-MEM was harvested and 20µl loaded onto a 12% SDS polyacrylamide gel for western blotting with anti-HA antibody. The remainder was used for the trypsin assay supplemented with 2ng/ml enterokinase, 1mM $CaCl_2$ and 5µM BZiPAR at pH 8. The reaction kinetics were followed over 60 minutes at 37°C (see section 2.9.2).

The supernatant harvested from cells transfected with pCEP wtPRSS1HA demonstrated a higher level of fluorescence achieved by cleaving the BZiPAR substrate in relation to the negative controls (see figure 6.8). This indicated that the enzymatic activity did not arise natively from the Opti-MEM medium or from transfection with the empty pCEP vector, but was likely related to the function of the secreted wtPRSS1HA protein. The western blot demonstrated a band corresponding to wtPRSS1HA detected in the harvested Opti-MEM medium only.

These results demonstrated that the wtPRSS1HA protein had enzymatic activity and was capable of cleaving the substrate BZiPAR (see figure 6.8). To ensure that this was also the case with PRSS1HA R122H and PRSS1HA N29I, transfection of HEK 293T cells was set up with the

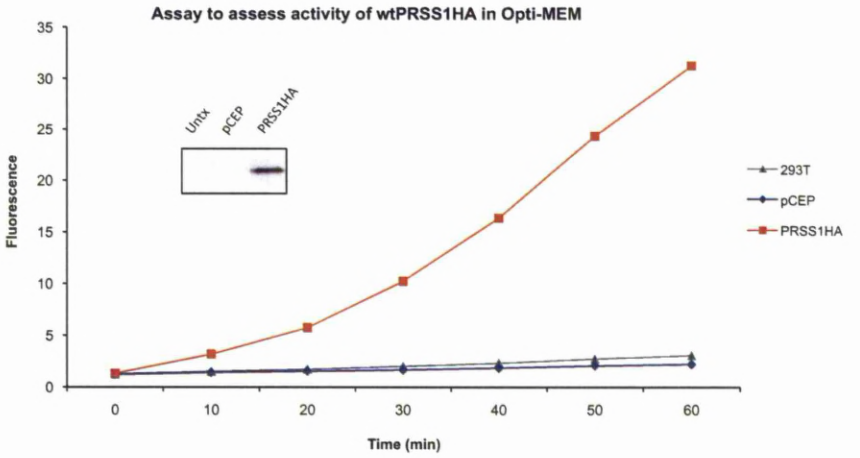

Fig 6.8 Assay to assess activity of wtPRSS1HA in Opti-MEM

Transiently transfected HEK 293T cells supplemented with Opti-MEM medium for 12 hours. The medium was harvested and 20μl loaded onto a 12% SDS polyacrylamide gel and subject to immunoblotting with anti-HA antibody (insert). The remainder of the Opti-MEM medium was supplemented with 2ng/ml enterokinase, 1mM $CaCl_2$, and 5μM BZiPAR (final concentrations) and the reaction kinetics followed over 60 minutes at 37°C. Opti-MEM harvested from untransfected cells (grey line) and mock transfected cells (blue line) were negative controls. The enzymatic activity of wtPRSS1HA (red line) was higher than in the controls. (Fluorescence measured in arbitary units).

wtPRSS1HA and the two mutant alleles. Untransfected HEK 293T cells and cells mock transfected with empty pCEP vector would serve as negative controls. All experiments were run in triplicates.

The assay confirmed that supernatant harvested from cells transfected with pCEP wtPRSS1HA had a significantly higher level of fluorescence by cleaving the substrate BZiPAR compared to the negative controls (see figure 6.9). However, the highest level of fluorescence was seen in supernatant harvested from cells transfected with pCEP PRSS1HA R122H whilst the fluorescence of supernatant from pCEP PRSS1HA N29I cells was lower in relation to the wild-type protein. The western blot revealed similar levels of all three proteins with no obvious asymmetry seen in the loading controls. The standard error of the mean was calculated for each triplicate with no large discrepancy or overlap (besides the two negative controls) seen. This result was seen repeated over multiple replications of the experiment.

The difference in fluorescence detected at 60 minutes between wtPRSS1HA and the mutants PRSS1HA R122H and PRSS1HA N29I indicated differing activity between the three enzymes in cleaving the substrate BZiPAR. This finding had not been described previously in the literature and raised the possibility that the effect may be due to the HA tag. We, therefore, repeated the assay using the untagged constructed to transiently transfect HEK 293T cells. pCEP wt PRSS1, pCEP PRSS1

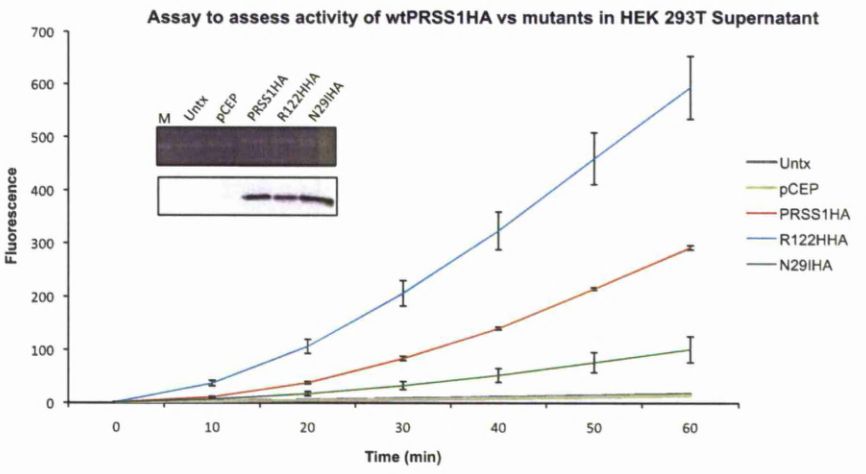

Fig 6.9 Assay to assess activity of wtPRSS1HA versus mutant alleles

Transiently transfected HEK 293T cells supplemented with Opti-MEM medium for 12 hours. The medium was harvested and 20μl loaded onto a 12% SDS polyacrylamide gel and subjected to immunoblotting with anti-HA antibody (insert; loading controls seen above). Harvested Opti-MEM medium was supplemented with 2ng/ml enterokinase, 1mM $CaCl_2$, and 5μM BZiPAR (final concentrations) and the reaction kinetics followed over 60 minutes at 37°C. Opti-MEM harvested from pCEP wtPRSS1HA (red line), pCEP PRSS1HA R122H (blue line) and pCEP PRSS1HA N29I show greater activity than the negative controls (pale lines; Untx, pCEP). Error bars show standard error of the mean

R122H and pCEP PRSS1 N29I were transiently transfected into HEK 293T cells and after 12 hours the culture medium was replaced by OptiMEM. A further 12 hours later the Opti-MEM was harvested and 20μl loaded onto a 12% SDS polyacrylamide gel for western blotting with anti-PRSS1 (13404) antibody. The remainder was used for the trypsin assay supplemented with 2ng/ml enterokinase, 1mM $CaCl_2$ and 5μM BZiPAR at pH 8 unless otherwise stated. The reaction kinetics were followed over 60 minutes at 37°C (as described above and in section 2.9.2).

The results from the untagged proteins demonstrated that given similar concentrations of protein, as shown by western blotting, there was no significant difference between the wtPRSS1 and the mutant PRSS1 R122H proteins in cleaving BZiPAR, as measured by the level of fluorescence. However the initial experiments showed that there was a lower level of fluorescence seen with relation to the mutant PRSS1 N29I protein (see figures 6.10 & 6.11). Interestingly, the peak fluorescence seen at 60 minutes with the untagged proteins was significantly higher than seen with the tagged proteins. The value was generally more than twice as great for the untagged proteins in similar conditions.

The experiments were repeated using the untagged proteins with variation in conditions to assess the impact of pH and calcium concentration (see figures 6.12-6.15). The supernatant was supplemented with either 1mM

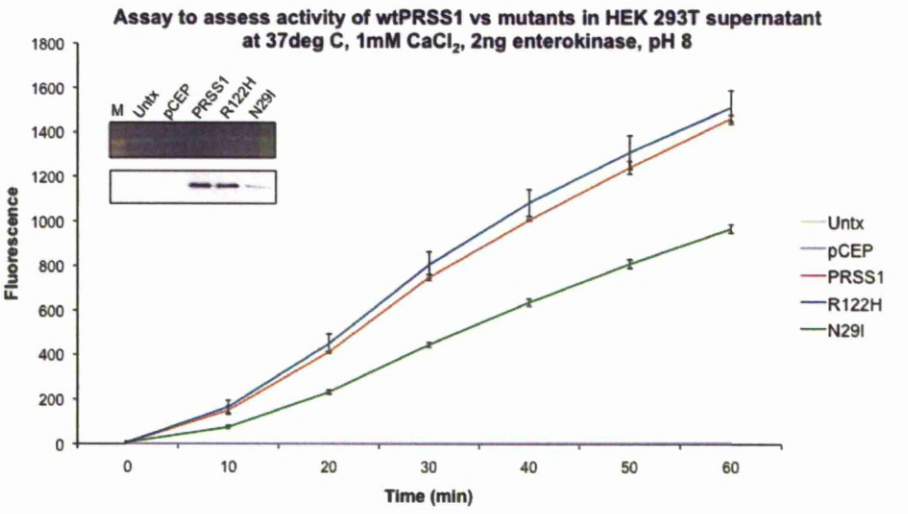

Fig 6.10 Assay to assess activity of wtPRSS1 versus mutant alleles

Transiently transfected HEK 293T cells supplemented with Opti-MEM medium for 12 hours. The medium was harvested and 20µl loaded onto a 12% SDS polyacrylamide gel and subjected to immunoblotting with anti-PRSS1 antibody (insert; loading controls seen above). Harvested Opti-MEM medium was supplemented with 2ng/ml enterokinase, 1mM $CaCl_2$, and 5µM BZiPAR (final concentrations) and the reaction kinetics followed over 60 minutes at 37°C. Opti-MEM harvested from pCEP wtPRSS1 (red line), pCEP PRSS1 R122H (blue line) and pCEP PRSS1 N29I show greater activity than the negative controls (pale lines; Untx, pCEP). Error bars show standard error of the mean (n=3).

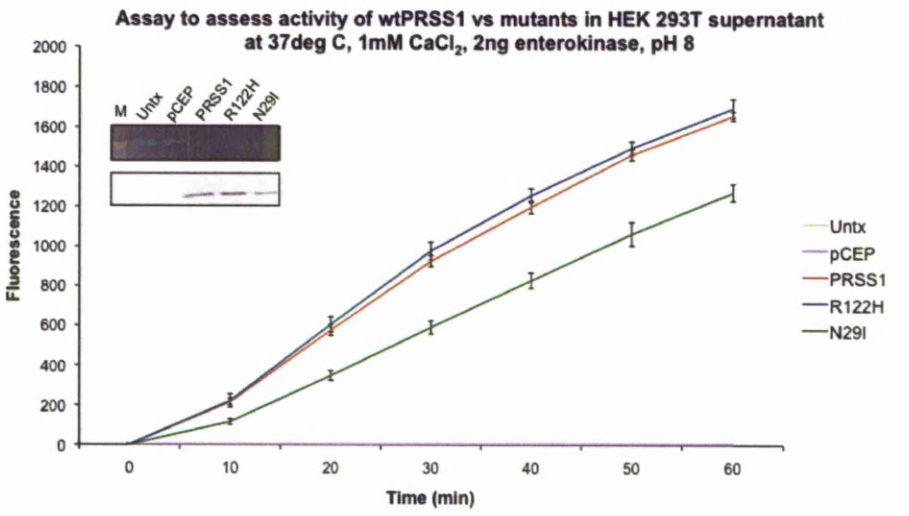

Fig 6.11 Assay to assess activity of wtPRSS1 versus mutant alleles

Transiently transfected HEK 293T cells supplemented with Opti-MEM medium for 12 hours. The medium was harvested and 20μl loaded onto a 12% SDS polyacrylamide gel and subjected to immunoblotting with anti-PRSS1 antibody (insert; loading controls seen above). Harvested Opti-MEM medium was supplemented with 2ng/ml enterokinase, 1mM $CaCl_2$, and 5μM BZiPAR (final concentrations) and the reaction kinetics followed over 60 minutes at 37°C. Opti-MEM harvested from pCEP wtPRSS1 (red line), pCEP PRSS1 R122H (blue line) and pCEP PRSS1 N29I show greater activity than the negative controls (pale lines; Untx, pCEP). Error bars show standard error of the mean (n=3).

$CaCl_2$ or 5mM EDTA and the pH of the OptiMEM was adjusted to either pH 8 or pH 5. The other conditions were maintained as described above.

No real difference was seen in terms of fluorescence between supernatant supplemented with 1mM $CaCl_2$ or 5mM EDTA, however, the level of fluorescence detected in samples with pH 5 was significantly reduced compared to similar experiments conducted at pH 8 (see figures 6.12-6.15). This suggested that under acidic condition (pH 5) the ability of the enzymes to cleave the substrate BZiPAR was significantly reduced. The PRSS1 N29I mutant had a lower level of fluorescence compared to wtPRSS1 and PRSS1 R122H, and this was particularly demonstrated in experiments conducted at pH 5 (see figures 6.14 & 6.15). Both wtPRSS1 and PRSS1 R122H had similar levels of fluorescence detected in the experiments, whether conducted at pH 5 or pH 8 and with 1mM $CaCl_2$ or 5mM EDTA (see figures 6.12-6.15).

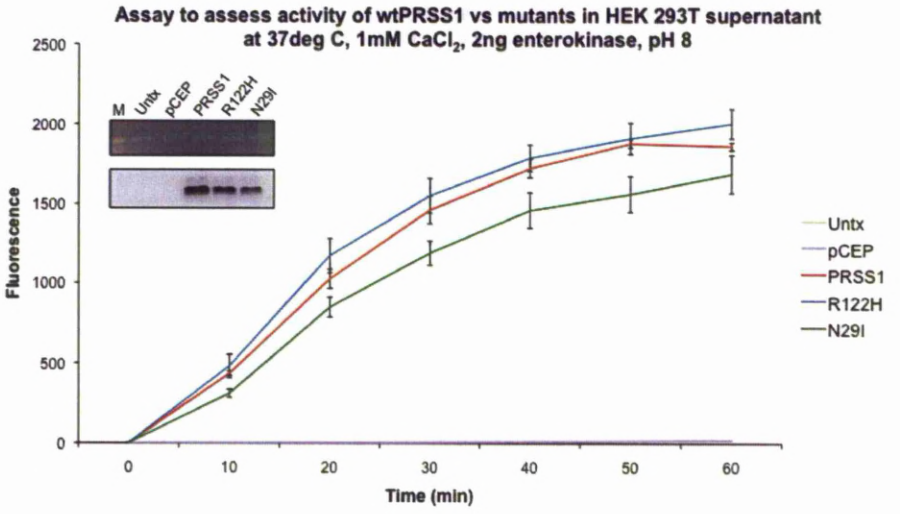

Fig 6.12 Assay to assess activity of wtPRSS1 at pH 8, 1mM $CaCl_2$

Transiently transfected HEK 293T cells supplemented with Opti-MEM medium for 12 hours. The medium was harvested and 20µl loaded onto a 12% SDS polyacrylamide gel and subjected to immunoblotting with anti-PRSS1 antibody (insert; loading controls seen above). Harvested Opti-MEM medium was supplemented with 2ng/ml enterokinase, 1mM $CaCl_2$, and 5µM BZiPAR (final concentrations) and the reaction kinetics followed over 60 minutes at 37°C. Opti-MEM harvested from pCEP wtPRSS1 (red line), pCEP PRSS1 R122H (blue line) and pCEP PRSS1 N29I show greater activity than the negative controls (pale lines; Untx, pCEP). Error bars show standard error of the mean (n=3).

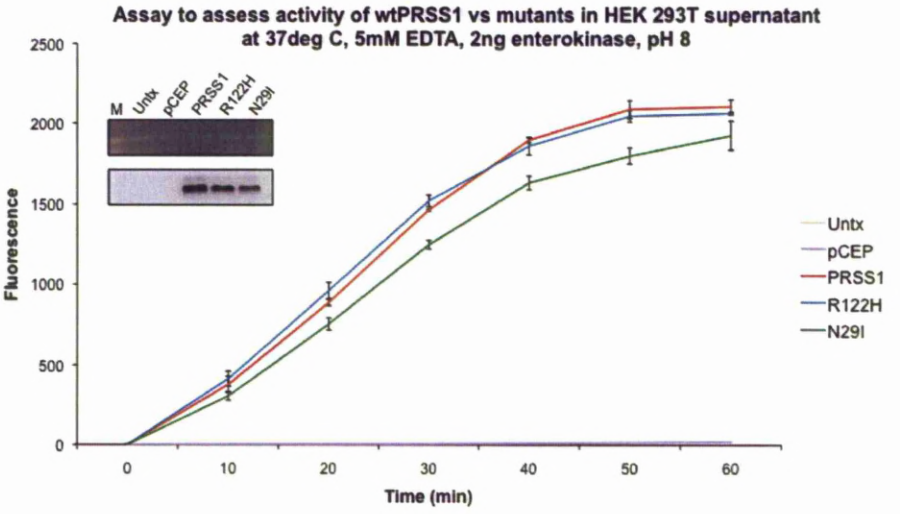

Fig 6.13 Assay to assess activity of wtPRSS1 at pH 8, 5mM EDTA

Transiently transfected HEK 293T cells supplemented with Opti-MEM medium for 12 hours. The medium was harvested and 20μl loaded onto a 12% SDS polyacrylamide gel and subjected to immunoblotting with anti-PRSS1 antibody (insert; loading controls seen above). Harvested Opti-MEM medium was supplemented with 2ng/ml enterokinase, 5mM EDTA, and 5μM BZiPAR (final concentrations) and the reaction kinetics followed over 60 minutes at 37°C. Opti-MEM harvested from pCEP wtPRSS1 (red line), pCEP PRSS1 R122H (blue line) and pCEP PRSS1 N29I show greater activity than the negative controls (pale lines; Untx, pCEP). Error bars show standard error of the mean (n=3).

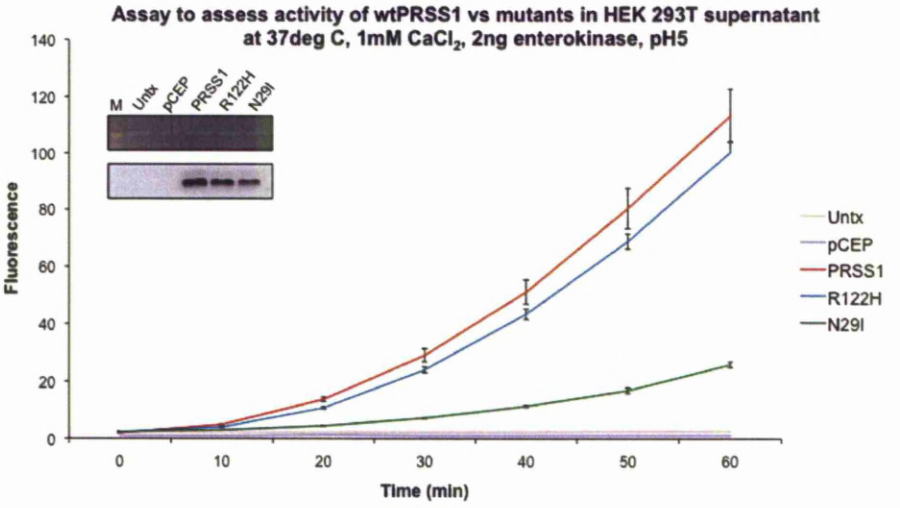

Fig 6.14 Assay to assess activity of wtPRSS1 at pH 5, 1mM $CaCl_2$

Transiently transfected HEK 293T cells supplemented with Opti-MEM medium for 12 hours. The medium was harvested and 20μl loaded onto a 12% SDS polyacrylamide gel and subjected to immunoblotting with anti-PRSS1 antibody (insert; loading controls seen above). Harvested Opti-MEM medium was supplemented with 2ng/ml enterokinase, 1mM $CaCl_2$, and 5μM BZiPAR (final concentrations) and the reaction kinetics followed over 60 minutes at 37°C. Opti-MEM harvested from pCEP wtPRSS1 (red line), pCEP PRSS1 R122H (blue line) and pCEP PRSS1 N29I show greater activity than the negative controls (pale lines; Untx, pCEP). Error bars show standard error of the mean (n=3).

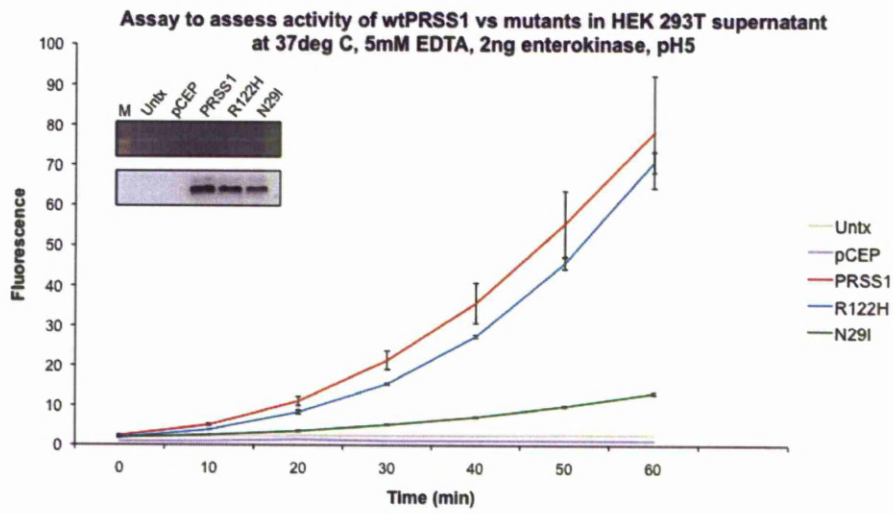

Fig 6.15 Assay to assess activity of wtPRSS1 at pH 5, 5mM EDTA

Transiently transfected HEK 293T cells supplemented with Opti-MEM medium for 12 hours. The medium was harvested and 20μl loaded onto a 12% SDS polyacrylamide gel and subjected to immunoblotting with anti-PRSS1 antibody (insert; loading controls seen above). Harvested Opti-MEM medium was supplemented with 2ng/ml enterokinase, 5mM EDTA, and 5μM BZiPAR (final concentrations) and the reaction kinetics followed over 60 minutes at 37°C. Opti-MEM harvested from pCEP wtPRSS1 (red line), pCEP PRSS1 R122H (blue line) and pCEP PRSS1 N29I show greater activity than the negative controls (pale lines; Untx, pCEP). Error bars show standard error of the mean (n=3).

6.3 Summary

Having generated plasmids designed to express either the wt human cationic trypsinogen or the R122H or N29I mutant forms it was necessary to assess *in vitro* expression and examine the that enzymatic function of each construct, with and without the HA tag. Due to the potential difficulty of identifying the transgenic protein from native murine trypsinogens a HA tag had been introduced on the C-terminus.

The pCEP-based expression vectors were used to transiently transfect H1299 cells and expression was assessed by western blot analysis. The anti-HA antibody and an anti-PRSS1 (13404) antibody were found to be the most sensitive at detecting, and therefore demonstrating, the *in vitro* expression of the proteins. The bands representing the HA tagged proteins were found to run higher than those for the untagged proteins, consistent with the higher molecular weight.

Attempts to assay the enzymatic activity of the proteins were initially unsuccessful. Transfected H1299 cells were lysed and loaded into 0.1M Tris-HCl with enterokinase and calcium chloride to activate the trypsinogen proteins. The fluorogenic substrate BZiPAR was used to assess the enzymatic activity of the trypsin. Despite numerous attempts, no activity was seen when compared to the negative controls. It was speculated that an inhibitor within the cell lysate might be affecting the assay and experiments using recombinant human trypsin found evidence of

this in H1299 cells as well as a variety of other cell lines in regular use in our laboratory.

At this stage personal communication with Miklos Sahin-Tóth about a forthcoming publication revealed that his team had transiently transfected human embryonic kidney 293T cell with human cationic trypsinogen and found the protein secreted into the culture medium (Sahin-Toth, Kukor et al. 2006). By removing the culture medium it was possible to harvest the proteins.

We used this technique to transiently transfect HEK 293T cells with our pCEP-based constructs and harvested the supernatant for the assay. The assay demonstrated cleavage of the BZiPAR substrate by the wtPRSS1HA protein, but interestingly, when comparing the activity of this protein to the two mutant alleles, we found that PRSS1HA R122H appeared to be more efficient at cleaving BZiPAR whilst PRSS1HA N29I had a reduced efficiency. There was no indication on the western blots to suggest that there was a significant difference in the relative quantities of each protein. No previous reports assessing the properties of cationic trypsinogen and the two mutant alleles had found any difference in their activity (Sahin-Toth 2001; Szilagyi, Kenesi et al. 2001), leading us to speculate whether the HA-tag, placed on the protein tail to aid identification, was having an influence on the enzymatic activity of one or more of the proteins.

Using the pCEP-based construct not carrying the HA-tag, we repeated the experiments. The assay showed that wtPRSS1 and PRSS1 R122H had similar levels of fluorescence indicating a similar ability to cleave the BZiPAR substrate whilst PRSS1 N29I appeared to be not as efficient. Interestingly, all the untagged proteins achieved a higher level of fluorescence at 60 minutes then seen with the HA-tagged proteins. This suggested that the HA-tag may influence the enzymatic activity of each of the proteins, though they retained a reduced ability to cleave the BZiPAR substrate.

A significant difference in the level of fluorescence was seen with respect to each protein when the experiments were conducted at pH 5 or pH 8. The acidic conditions reduced the level of fluorescence of the wtPRSS1 and the PRSS1 R122H proteins almost 20-fold at 60 minutes, whilst fluorescence of the PRSS1 N29I protein was reduced almost 100-fold.

Chapter 7

7 SUMMARY AND DISCUSSION

7.1 Introduction

Hereditary pancreatitis is an autosomal dominant disease, characterised by recurrent episodes of acute pancreatitis usually beginning during adolescents and progressing to chronic pancreatitis. It was first described in 1952 by Comfort and Steinberg (Comfort and Steinberg 1952) but it was not until 1996 that the disease-causing gene was mapped to chromosome 7q35, implicating mutations of the cationic trypsinogen gene (Whitcomb, Gorry et al. 1996). The disease is clinically characterised by episodes of acute pancreatitis begin in adolescence, and often progressing to chronic pancreatitis, with patients having a significantly increased risk of developing pancreatic adenocarcinoma (Howes, Lerch et al. 2004; Rebours, Boutron-Ruault et al. 2008).

The two most common mutations of cationic trypsinogen, R122H and N29I, have been well characterised in patients with hereditary pancreatitis. However, the pathophysiology of these mutations and the general pathways leading to pancreatitis and adenocarcinoma remain poorly understood. In this respect hereditary pancreatitis is unique as it allows interrogation of all three common pathologies of the exocrine pancreas.

The ultimate aim of this work was to generate a genetically modified mouse model that stably expressed either wild type or mutant human cationic trypsinogen, in the acinar cells of the pancreas. The transgene would be integrated into the murine genome in a random fashion and have constitutive expression based on an upstream rat elastase promoter or require doxycycline induction as part of a Tet-On compound inducible mouse system. These models would enable us to assess whether the presence of human cationic trypsinogen, in its wild type or mutated form, in the mouse pancreas results in a pathological phenotype, and if so, whether it is consistent with the clinical spectrum seen in patients with hereditary pancreatitis.

7.2 Generation of mice with inducible transgene expression

The primary aim was to generate genetically modified mice with inducible expression of the transgenes, based on the Tet-On system, specifically within the acinar cells of the pancreas. This transgenic model allows precise spatial and temporal control over the expression of the genes of interest. In order to achieve this aim two constructs were generated; the first inducible construct, based on the pBI-G bidirectional vector, carries the gene of interest i.e. wt PRSS1 or one of the two mutants and the second construct, which expresses the inducer/regulator protein OptrtTA, driven by an upstream rat elastase 1 promoter to ensure tissue specificity. Cross breeding these two strains of mice would produce a compound mouse that

would express OptrtTA specifically within the acinar cell by virtue of the rat elastase promoter. Upon feeding the compound mouse with doxycycline, a conformational change would take place, resulting in binding of the OptrtTA to the Tet-responsive element (TRE) and ignition of transcription of the gene of interest in the acinar cells of the pancreas.

Founder animals were identified for all the PRSS1-based transgenes. These animals were back-crossed to generate larger numbers of stable heterozygous animals and ultimately homozygous animals for each transgene.

Generation of mice with integration of the pBEG OptrtTA transgene into the genome was far less successful. Despite the creation of a relatively large number of potentially positive animals, screening failed to identify integration of the transgene into the murine genome. This failure was of great concern, as the pBEG OptrtTA transgene would be crucial in driving expression of the PRSS1 transgenes in the final compound mouse. Without this transgenic strain it would be impossible to induce the expression of the PRSS1-based transgenes. Due to these difficulties, the plasmid was redesigned and cloned again prior to microinjection. The sequencing revealed that there were two *Eco*NI site located within the transgene and digesting the murine genome with this restriction endonuclease resulted in a fragment that would not run at the expected position, and may in fact be too small to detect, on a Southern blot. New animals were created by

microinjection of the new pBEG OprtTA transgene and the restriction endonuclease *Stu*I was used to digest the murine genome. This strategy led to the identification of a positive founder animal. During the course of this work, a group described a similar transgenic animal that they had generated expressing the rtTA protein under the control of a rat elastase promoter (Baumann, Wagner et al. 2007). If our own pBEG OptrtTA transgenic mouse did not express the rtTA protein or was unsuitable for the intended experimental work, we would endeavour to import this transgenic animal.

7.3 Generation of mice with constitutive transgene expression

As a consequence of the difficulties encountered in establishing a mouse carrying the pBEG OptrTA transgene, a critical component of the inducible model, a different strategy of generating genetically modified mice stably expressing human cationic trypsinogen was developed. The genetically modified mice in which the rat elastase promoter driven genes of interest were integrated into the murine genome would allow constitutive expression of the PRSS1 based transgenes within the acinar cells of the mouse pancreas. Though this model would not allow temporal control, it retained the crucial spatial specificity of limiting transgene expression to the acinar cells of the mouse pancreas. This model had potential limitations, as it would possibly allow expression of human cationic trypsinogen during foetal development, a consequence of which

may be pathological changes in the pancreatic parenchyma seen from birth in the transgenic animals or potentially a fatal phenotype as seen in the *spink3* null mice (Ohmuraya, Hirota et al. 2005). Although these findings would be of immense interest, further interrogation of the adult mouse pancreas would become limited. Due to these concerns the inducible model became our primary strategy whilst the constitutive expression model was initially envisaged as a back up project.

Development and identification of founder animals for both the inducible and constitutive transgene expression models were completed during my tenure in the laboratory. Work to generate homozygous animals for each strain and then analyse the resultant phenotype, which developed both spontaneously and upon treatment with caerulein, took place after this period of time and is outside the scope of this thesis.

7.4 Trypsin Assay

Whilst generating a transgenic mouse model to analyse the role of human cationic trypsinogen *in vivo,* experiments were also undertaken to assess the enzymatic activity of wild type and mutant trypsin *in vitro*. Several pCEP-based expression constructs were generated to examine the expression and enzymatic activity of untagged and HA-tagged human cationic trypsinogen and the mutant alleles *in vitro*. This was a critical question, as transcription of the transgenic DNA and the confirmed

presence of a protein product within the acinar cells of the murine pancreas could be no guarantor of an enzymatically functional protein. If eventual analysis of the transgenic mice revealed no difference in phenotype, compared to non-transgenic mice, then the question would be even more pertinent.

The pCEP-based expression constructs were initially used to transiently transfect H1299 cells and expression was analysed by western blot analysis using anti-HA antibody and an anti-PRSS1 (13404) antibody. Even though expression of the PRSS1 proteins was detected, cellular lysate of these transfected cells did not seem to possess any enzymatic activity as I failed to detect any fluorescence indicating cleavage of the substrate BZiPAR using an *in vitro* trypsin activity assay. This failure to detect trypsin activity raised questions as to whether there was insufficient trypsin in the lysates to enable a detectable response in the assay, whether post-translational misfolding of the proteins abolished the enzymatic pocket, or if other proteins present in the cell lysate may have inhibited the enzymatic ability of cationic trypsin.

Whilst addressing these questions, a paper describing a cell line, human embryonic kidney 293T (HEK 293T), amenable to transient transfection with cationic trypsinogen, secreted the protein into the culture medium (Sahin-Toth, Kukor et al. 2006). We decided to adopt this method to

investigate the enzymatic activity of cationic trypsin and the two mutant alleles.

The pCEP-based plasmids were again used to transiently transfected human embryonic kidney 293T cell, which appeared to secrete the protein into the tissue culture medium. The culture medium/supernatant was harvested for the assay. The modified assay demonstrated cleavage of the BZiPAR substrate by the wtPRSS1HA protein, thus confirming for the first time, both the expression and enzymatic activity of the construct. However, when comparing the activity of the wt protein to the two mutant alleles, we found that PRSS1HA R122H appeared to be more efficient at cleaving BZiPAR whilst PRSS1HA N29I had a reduced efficiency. The bands corresponding to the proteins on the western blots were of similar intensity suggesting that there was no significant difference in the relative quantities of each protein. No previous reports assessing the properties of cationic trypsinogen and the two mutant alleles had found any difference in their activity (Sahin-Toth 2001). However, an early paper investigating the properties of the N29I mutation had found that wt human cationic trypsinogen was activated 2-fold faster than the N29I mutant by bovine enterokinase (Sahin-Toth 2000). The implications of this finding were not discussed in the paper and it was not raised again in the future studies reported by the group.

These findings led us to speculate whether the HA-tag, placed on the C-terminus of the three trypsinogen proteins to aid identification, was having an influence on the enzymatic activity of one or more of the proteins. To resolve this issue we sort to repeat the experiments using the pCEP based constructs without the HA-tag, which were therefore faithful replications of cationic trypsinogen and its two common mutations as found in humans.

The assay showed that wtPRSS1 and PRSS1 R122H had similar levels of fluorescence indicating a similar ability to cleave the BZiPAR substrate whilst PRSS1 N29I appeared to be not as efficient. A significant difference in the level of fluorescence was seen with respect to each protein when the experiments were conducted at pH 5 or pH 8. The acidic conditions reduced the level of fluorescence of the wtPRSS1 and the PRSS1 R122H proteins almost 20-fold at 60 minutes, whilst fluorescence of the PRSS1 N29I protein was reduced almost 100-fold. This is consistent with findings that an acidic pH stabilises pancreatic zymogens (Colomb and Figarella 1979)

The results from the assay analysing the untagged proteins were similar to those reported in the literature (Sahin-Toth and Toth 2000; Sahin-Toth 2001) though the reduced activity of the N29I mutant is not adequately explained. An early report investigating the influence of the N29I mutation on human cationic trypsinogen had found that it resulted in a 2-fold reduction in activation by bovine enterokinase (Sahin-Toth 2000).

However the catalytic activity was found to be similar to the wt human cationic trypsin and the focus of the paper turned to the increased autoactivation of the N29I mutant protein in acidic conditions. This has become accepted as the mechanism underlying the pathophysiology of acute pancreatitis seen in patients carrying the N29I mutation.

Our findings of a decrease in the ability of PRSS1 N29I to cleave the substrate BZiPAR in comparison to wtPRSS1 and PRSS1 R122H raises the prospect that the N29I mutation may reduce enzymatic activity. Alternatively, the effect may be due in part to a decreased activation of PRSS1 N29I by human enterokinase resulting in a time lag reflected by reduced levels of fluorescence. However, as a mechanism for causing hereditary pancreatitis the role of this finding is not clear and further work is required to delineate whether the reduced activity is real or an artefact.

Interestingly, all the untagged proteins achieved a higher level of fluorescence at 60 minutes then seen with the HA-tagged proteins. This suggested that the HA-tag may influence the enzymatic activity of each of the proteins, and though the ability to cleave the substrate BZiPAR was reduced in those proteins with the HA tag it was not completely abolished.

This finding may have an influence on the eventual phenotype of the transgenic mice developed during this project, as reduced activity of the

enzymes may manifest as a mild insult to the acinar cells of the murine pancreas that may or may not be amenable to clinical characterisation.

7.5 Discussion & Future work

Despite numerous set backs and difficulties, we were finally able to generate and positively identify founder animals for both the inducible mouse model and the constitutive expression model of hereditary pancreatitis. The transgenes were successfully integrated into the murine genome and further generations of positive animals were generated.

The *in vitro* trypsin assay demonstrated that the PRSS1-based transgenes, with the HA tag, were expressed in cultured human cell lines and were also functionally active enzymes capable of cleaving the substrate BZiPAR. Interestingly, there was an unexpected difference in the apparent enzymatic activity of the wild type PRSS1HA compared to the two mutant alleles. We consistently observed that the mutant PRSS1HA R122H was more active than the wild type protein whilst the mutant PRSS1HA N29I appeared to be less active. The difference in activity observed may be due to subtle differences in the relative concentrations of the individual proteins present in the assayed supernatant, though this should have become apparent from successive replications of the assay. Another possibility is the addition of the HA tag, intended to aid protein identification from amongst the native murine trypsinogens in the mouse pancreas, results in minor alterations of the tertiary structure of the zymogens based on the

interactions of the HA tag in combination with the wild-type or mutated amino acids. These modifications may lead to the variation in enzymatic activity as seen in the experiments involving the cleavage of the substrate BZiPAR.

When the experiments were repeated with human cationic trypsinogen or the mutant proteins without the HA-tag, wtPRSS1 and PRSS1 R122H demonstrated a similar ability to cleave the BZiPAR substrate under various conditions. However, PRSS1 N29I appeared to have a reduced ability to cleave BZiPAR under similar conditions, as had been seen in the experiments analysing the proteins with the HA-tag.

The finding that the level of fluorescence detected from the cleavage of the BZiPAR substrate was reduced in proteins with the HA-tag raised questions regarding the potential implications this may exert in the transgenic animals. Though there are multiple permutations that can be described with regards to the possible effects on the phenotype of the transgenic animals, the primary concern was that a reduced level of enzymatic activity may significantly ameliorate the pathological insult on the acinar cells of the murine pancreas. The animals may demonstrate no evidence of pancreatitis or only a mild phenotype. Alternatively, the enzymatic activity may still be sufficient that if the zymogens are inappropriately activated within the acinar cells, then inflammatory pathways are activated leading to acute and/or chronic pancreatitis.

The biochemical kinetics, previously described by Sahin-Tóth and colleagues (Sahin-Toth 2001) when describing the R122H and N29I mutations, would also be amenable to investigation using the trypsin assay. This would allow analysis of autoactivation and autolysis of recombinant human cationic trypsinogen, and the R122H and N29I mutants, transcribed and processed by cultured human cells as opposed to E.Coli. The previous work carried out by Sahin-Toth *et al* in characterising the biochemical properties of these proteins relied on transcription and post-translational modification of the proteins in E. Coli and then refolding the protein *in vitro* after extraction by sonication. There exists the real possibility that the final proteins are similar but not identical to human expressed cationic trypsinogen, rendering a discrepancy in the biochemical findings.

The trypsin assay was developed to examine the ability of our antibodies in detecting expression of cationic trypsinogen and indeed demonstrate that the plasmids generated were able to allow expression of the genes in transiently transfected cells *in vitro*. Once expression of the proteins had been verified, we sought to confirm that the proteins were functional as enzymes before moving onto interrogation of the transgenic animals. Further experiments to understand the biochemistry of the proteins had to take a minor role to the primary aim of generating and analysing the transgenic animals. However, this work deserves greater emphasis in the

near future to determine whether there is a real difference in the enzymatic activity of wild cationic trypsinogen and the mutant alleles.

With regards to the genetically modified mice, all positively identified animals from both the inducible and constitutive expression models are due to be extensively backcrossed to generate a large pool of transgenic animals for each of the individual strains. Eventually, compound mice would be generated for the inducible model, carrying the pBEG OptrtTA transgene and one of the PRSS1-based transgenes. In the event of continued difficulties arising from the pBEG OptrtTA animals, we would look to import a similar transgenic animal that had been validated (Baumann, Wagner et al. 2007), as part of collaboration with another laboratory. Animals from both models would be dated at birth and eventually analysed at different time points to determine whether they develop a phenotype, either spontaneously or after a pharmacological challenge, characteristic of pancreatitis.

No group had developed a transgenic animal with expression of wild type human cationic trypsinogen or contrasted this against animals carrying the common hereditary pancreatitis associated mutations at the onset of this work and this remains the case at the time of writing. One group has, however, during the course of our work, described a transgenic mouse model expressing human cationic trypsinogen carrying the R122H mutation (Selig, Sack et al. 2006). What are the consequences, if any, of

expressing human cationic trypsinogen in the murine acinar cell? Would there be a different phenotype between the wild type and mutant forms of human cationic trypsinogen, or would the model simply result in over-expression of trypsin regardless of the mutations? Ultimately, would our hopes of seeing the full spectrum of hereditary pancreatitis in these transgenic animals be a naive assumption?

The evidence from previous studies of transgenic animals has taught us that the pathophysiological response to a genetic modification rarely gives the expected phenotype. Given the species variation and the redundancy built into cellular pathways that has now become apparent, this is not a surprise. The initial attempts to understand the role of p53 in epithelial cancer led to the development of a transgenic mouse model over-expressing mutant alleles of p53 (Lavigueur, Maltby et al. 1989). The transgenic animals were found to have a high risk of lymphomas and sarcomas rather than the expected epithelial cancers. Other groups have sort to assess the function of a particular gene or protein by generating a knock out mouse, only to find that the genetic modification leads to a lethal phenotype (Ohmuraya, Hirota et al. 2005).

These concerns were part of the reason that we opted to develop an inducible model to allow temporal, as well as spatial, control of transgene expression. In this way we could assess if there were effects on the pancreas at the time of birth of the transgenic animals, or indeed if

transgene expression was a lethal condition, whilst also having the option of activating expression of the transgene in an adult mouse to study the resultant phenotype. As we were using the elastase promoter, expression of the transgene in the constitutive model could begin at embryonic day 14, consistent with expression of the elastase protein (Ornitz, Hammer et al. 1987).

The importance of transgenic human cationic trypsinogen is found in its propensity to autoactivate relative to trypins from other species (Buck, Bier et al. 1962; Colomb and Figarella 1979). The transgenic mice carrying the wild type human cationic trypsinogen may not only function as a control group to assess the impact of the disease associated mutants, but may provide a new model for experimental acute and/or chronic pancreatitis. Though there is a wide range of models available to interrogate the cellular mechanisms leading to pancreatitis, none have proven fruitful in the face of developing effective preventative or therapeutic strategies (Steinberg and Schlesselman 1987). A study comparing the outcomes of therapeutic studies in animal models of acute pancreatitis found that 81% of the 25 animal protocols studied had a positive outcome with respect to survival whilst only 7.7% of the resulting 13 human studies demonstrated a positive outcome (Steinberg and Schlesselman 1987). Many investigators believe that the fundamental problem lies in the artificial methods employed to induce pancreatitis in these animal models. A model that has a spectrum of

disease closely mimicking that seen in human pancreatitis may be an avenue to developing successful preventative and therapeutic medicines.

Though it is impossible to predict the phenotype that will eventually be exhibited by these transgenic animals, it is still possible to speculate and rationalise the potential of the model. In an ideal world the model would recapitulate the full spectrum of disease as seen in human patients with hereditary pancreatitis. In this scenario, the transgenic animal carrying the R122H or N29I mutant alleles would develop acute pancreatitis early in life, with a large proportion developing histological evidence of chronic pancreatitis with increasing age and ultimately a significant number then exhibiting pancreatic adenocarcinoma. It almost goes without saying that such a model would be highly sought after amongst the research community as a very powerful tool for dissecting the pathways underlying the disease mechanisms and driving the progression from acute to chronic pancreatitis and cancer.

On the other hand, the simple presence of human cationic trypsinogen, whether wild type or mutant may be a significant insult to the murine pancreas and result in either recurrent episodes of acute pancreatitis or chronic pancreatitis. In this respect, the constitutive expression model may show little or no significant difference between the phenotype of the three strains of transgenic animal, in effect behaving like an overexpression model of trypsinogen. This model would have great utility in studying the

role of trypsinogen as an early player in the pathogenesis of pancreatitis and aid understanding of the cellular response to excess trypsinogen and how this influences cell fate with regards to apoptosis or necrosis. The inducible model would be a powerful tool in this setting, as it would allow precise control of trypsinogen expression for a defined time period, after which the animals could be analysed. Temporal control of trypsinogen expression could allow investigators to identify the site of trypsinogen activation, mechanism of injury, induction of inflammatory mediators and quantify the efforts of a cell's protective machinery to limit the subsequent injury. These models would sit alongside the current models of experimental acute pancreatitis, and allow interrogation of the earliest events in acute pancreatitis from a different standpoint. Recent work, utilising a CMV driven construct expressing a mutant trypsinogen that was activated by an intracellular protease, PACE (paired basic amino acid cleaving enzyme), demonstrated that expression of intracellular trypsin induced apoptosis and did not activate the inflammatory mediator NF-κB (Ji, Gaiser et al. 2009). Indeed, the extracellular presence of trypsin proved to be a powerful inducer of NF-κB. This study claimed to be the first direct evidence of the biological effects of intracellular trypsin activity on pancreatic acinar cells, and if validated in our research may yield some interesting results.

There is a reasonable probability that the transgenic animals fail to produce a spontaneous or pharmacologically induced phenotype. The underlying reason for this may range from human cationic trypsinogen and the mutant alleles being very well tolerated and therefore not pathological in the murine pancreas through to inadequate expression of the transgenes to allow a phenotype to develop. The only model to date that describes integration of a mutant human cationic trypsinogen transgene into the murine genome appears to have suffered from a problem with low transgene expression levels (Selig, Sack et al. 2006). The cellular response to an injurious transgenic human cationic trypsinogen may be to down regulate the gene and thus prevent further damage or initiate apoptosis, limiting the acinar population to cells with low transgene expression. In this scenario, it would be difficult to demonstrate whether the transgenes may have had a pathological impact given strong expression. There may be an opportunity to compare the constitutive expression model against the inducible model to analyse whether transgene expression levels are a temporally dependent phenomenon. As discussed above, Ji *et al* demonstrated that intracellular trypsin resulted in acinar cell apoptosis and did not activate an inflammatory response via NF-κB (Ji, Gaiser et al. 2009). The *in vivo* implication of these findings when extrapolated to our models may mean that no phenotype consistent with acute or chronic pancreatitis is identified, and instead we see animals with various degrees of pancreas atrophy.

The only successful genetically modified mouse model to date, that has attempted to assess the *in vivo* effects of the R122H mutation showed an early onset of acinar cell injury and inflammatory cell infiltration, progressing to pancreatic fibrosis with increasing age (Archer, Jura et al. 2006). Despite showing that persistent pancreatic injury may be linked to chronic pancreatitis, it was not clear from the data whether the injury was related to the R122H mutation introduced into one of the murine trypsinogens or a consequence of trypsinogen overexpression that overwhelmed the acinar cell's protective mechanisms. It certainly had little to say with regards to how the murine pancreas may handle the presence of human cationic trypsinogen and therefore limits the potential implications for our models.

Invariably, once analysis of the transgenic mouse models is underway there will be surprise findings that one could not anticipate, which lead to new and unexpected avenues of research. It will be interesting to see what future research tells us about the role of cationic trypsinogen in the early events of acute pancreatitis and how this leads to chronic pancreatitis and in particular it will be interesting to see how the genetically modified animals generated in this thesis are used to answer these questions.

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